

ENZYMATIC PROTEIN CROSSLINKING IN WHEAT-BASED FOODS

*A thesis submitted in partial fulfilment of the requirements
for the degree of*

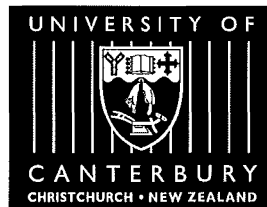
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by

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“Come, lay your trophies at His feet, and crown Him Lord of All”

- E. Perronet,
1780

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Abstract

This research investigated the use of the enzymes transglutaminase (TGA) and glucose oxidase in bread and croissants, in order to enhance their functional properties and baking performance. The means by which these enzymes are thought to act is by the crosslinking of specific proteins of wheat flour. Whereas glucose oxidase reportedly improves baking quality through the addition of disulfide crosslinks to gluten proteins, TGA catalyses the formation of ϵ -(γ -glutamyl-lysine) crosslinks.

SDS-PAGE and HPLC enabled the identification of the changes caused by the crosslinking reactions. Crosslinking of the wheat proteins by TGA occurred during dough mixing, with no further effects apparent with proofing or with increased enzyme concentration. Long-term frozen storage of croissant doughs maintained the initial effect of adding TGA to the croissant formulation.

The proteins most dramatically affected by TGA were the large, insoluble glutenins, the proteins responsible for the strength and elasticity of dough. The gliadin proteins were apparently unchanged by TGA *in situ*, although in *in vitro* studies the gliadins were crosslinked by TGA, leading to the novel conclusion that the gliadins appear to be inaccessible for crosslinking while within the polymeric gluten complex. The albumins and globulins were also shown to be crosslinked by TGA and it appears that this contributed to the reported improvements in crumb strength and texture.

Studies were then conducted using glucose oxidase as the crosslinking agent in bread and croissants. Molecular studies showed the effect of disulfide crosslinking on the glutenins to be far less than with TGA, thus explaining the lack of any observed increase in product volume. However, improvements to crumb texture could be explained by the crosslinking of the albumins and globulins by glucose oxidase. The occurrence of non-disulfide crosslinks was demonstrated in specific protein fractions from glucose oxidase-treated doughs, in contrast to the literature. Thus glucose oxidase was found to crosslink the

albumins and globulins with both disulfide and non-disulfide crosslinks, and the glutenins with some non-disulfide crosslinks.

Measurements of lysine upon crosslinking was tested as a means to measure rate of the TGA reaction. An existing assay, which measures the formation of hydroxamate by TGA, was developed for use in dough. A new method was developed for the measurement of TGA activity using computer image analysis software. Three model proteins, bovine serum albumin (BSA), ribonuclease (RNase A) and κ -casein were tested for crosslinking rates by TGA and the measured rates were related to literature reports. This method was then used to measure the crosslinking rate of a high molecular weight subunit of glutenin, by TGA.

Significant insights were gained from this research regarding the crosslinking of wheat proteins. These findings are likely to have major implications for the development of enzymatic improvers in baked products.

Abbreviations

A	absorbance units
A ₃₄₀	absorbance reading at 340 nm
A ₃₄₃	absorbance reading at 343 nm
A ₅₉₅	absorbance reading at 595 nm
A ₅₂₅	absorbance reading at 525 nm
AMPS	ammonium persulfate
BSA	bovine serum albumin
¹³ C	carbon 13
°C	degrees Celsius
CBZ	benzyloxycarbonyl
Cys	cysteine
dH ₂ O	distilled water
DHA	dehydroascorbic acid
DTT	dithiothreitol
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl
FNDP	1-fluoro-2,4-dinitrobenzene
g	grams
gln	glutamine
glu	glutamate
gly	glycine
GODIC	2-ammonio-6(-[2-[(4-ammonio-5-oxido-5-oxopentyl)amino]-4,5-dihydro-1H-imidazol-5-ylidene]amino)hexanoate

^1H	hydrogen
H-bonds	hydrogen bonds
HPLC	high performance liquid chromatography
HMW	high molecular weight
kDa	kilodaltons
L	litres
LMW	low molecular weight
L_n	Natural logarithm
Lys	L-lysine
M	molar
mA	milliamps
MALDI	matrix-assisted laser desorption ionisation
MDD	mechanical dough development
μg	microgram
μl	microlitres
μM	micromolar
mg	milligrams
mL	millilitres
mM	millimolar
NAD^+	oxidised nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
nm	nano metres
ODU	optical density unit(s)
OPA	<i>o</i> -phthaldialdehyde
PAGE	polyacrylamide gel electrophoresis

PDI	protein disulfide isomerase
p.p.m	parts per million
R ²	regression coefficient
RNAse A	ribonuclease A
RP-HPLC	reverse-phase high performance liquid chromatography
r.p.m	revolutions per minute
s	seconds
SDS	sodium dodecyl sulfate
SE-HPLC	size-exclusion high performance liquid chromatography
sem	standard error of the mean
sp.	species
TEMED	N, N, N',N'-tetramethylethylenediamine
TFA	trifluoroacetic acid
TGA	transglutaminase
TNBS	trinitrobenzenesulfonic acid
Tris	Tris (hydroxymethyl) aminomethane
v/v	unit volume per unit volume
WH/kg	work hours per kilogram
WRI fat	Wheat Research Institute fat
w/v	unit weight per unit volume

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Chapter One

INTRODUCTION

1.1 Proteins and their role in foods

1.1.1 The importance of proteins in food systems

Proteins are a diverse family of macromolecules that are of major importance in living systems. They perform a variety of roles, from forming the basic structural building blocks of cells and tissues to regulating the function of the whole system through enzymatic and hormonal processes. Proteins such as collagen, elastin and keratin provide many tissues and organs with features vital to their structure, while other proteins have roles in catalysis (e.g. enzymes), transport (e.g. haemoglobin) and defence (e.g. immunoglobulins and allergens) (Voet et al, 1999).

Proteins are polymers of L- α -amino acid residues, which are linked by amide (peptide) bonds. The structure of a particular protein is defined by the amino acid sequence at the primary level and the three-dimensional conformation at the secondary, tertiary and quaternary levels of organisation (Mathews et al, 2000).

In foods, proteins are of importance, not only for their nutritive value, but also for the features that determine particular consumer sensory properties. For example, the textural properties of bakery products are due to the viscoelastic nature of wheat gluten proteins; the texture and succulence of meat are the result of unique muscle proteins and in dairy products the curd-forming proteins give rise to the colloidal structure of casein micelles (Damodaran, 1997). In addition to these, characteristics such as solubility, viscosity, emulsification and product volume are often a direct reflection of the protein composition of the food. Such qualities, known collectively as functional properties, were first defined by Kinsella (1976) as “those physical and chemical characteristics, which determine how the food performs during preparation, processing and storage”.

1.1.2 The structure-function relationship of food proteins

The specific chemical and biological properties of a protein are, to a large extent, controlled by its structure. The native three-dimensional conformations of proteins such as enzymes, for example, are known to be crucial for their biological activity. In foods, functional properties are the direct result of the structural features of the component proteins, including size, shape, amino acid composition and sequence, as well as charge density and distribution (Boye and Ma, 1997).

In as much as the structure of a protein determines its functionality, it follows that an alteration to the structure of a protein may result in changes to its inherent biological and chemical properties. This can be exploited in processed foods, where the structure of food proteins may be deliberately altered in order to achieve desired functional properties.

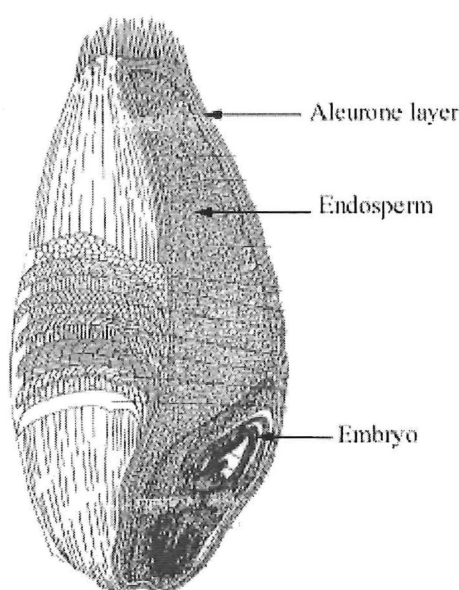
The mechanisms by which structural changes in proteins alter their properties in foods are not well understood. There has been a growing need to understand this relationship, arising from more sophisticated processing brought about by automation and from the ever-increasing range of foods with special requirements. A knowledge of the precise relationship between structure at the molecular level and functionality in food proteins potentially provides the food scientist with a very useful tool with which to manipulate food properties. This is especially so because food proteins in their natural state do not always possess optimal functional properties. They are, therefore, often modified structurally, in order to bring about the necessary improvements for greater consumer appreciation (Hamada, 1992 a). Indeed, a major task of modern food technology is the generation of new structures that have acceptable characteristics from a limited range of ingredients (Dickinson, 1997). It is with such modifications that this research is concerned, with regard to the proteins of wheat flour.

1.2 Importance of proteins in wheat-based foods

1.2.1 Source of wheat protein

Wheat is the largest and most versatile of the world's cereal crops. Most of the wheat grown for human consumption is *Triticum vulgare* or *Triticum aestivum*, while a closely related species, *Triticum durum*, is used for pasta products and some breakfast cereals (Frazier, 1992). Wheat flour is made by grinding and separating the starchy endosperm of the mature grain (figure 1.1) from the outer and inner seed coats and the aleurone layer. This flour is then combined with water to make a dough (or batter) from which wheat-based products are manufactured.

Figure 1.1: The mature wheat kernel (modified from Bushuk, 1998). The starchy endosperm with its associated proteins serves as the nutritional source for the growing embryo. The aleurone layer is removed with the seed coats as bran, during milling.



Bread flour is essentially pure endosperm, which consists of cells packed with starch granules embedded in a largely protein matrix. Starch makes up about 70-80% of the dry kernel weight, while proteins make up 10-15% and other constituents, such as lipids and non-starch polysaccharides (cell wall fragments), make up 1-2% of the dry weight of the grain (Shewry and Tatham, 1997).

Despite their relatively small percentage in the grain, proteins are recognised as being responsible for many of the quality parameters that are associated with wheat-based products. The unique viscoelastic and gas-retaining properties of wheat-based doughs, among other properties that make it suitable for a variety of products, are attributed mainly to this protein component (Bushuk, 1985).

Wheat proteins include those with enzymatic activity and those involved in forming the complex gluten network. The mixture of the proteins present is extremely complex, with much heterogeneity within each class. The relative amounts of the different proteins alone cannot explain all the variations in breadmaking quality (Weegels et al, 1996; MacRitchie and Lafiandra, 1997). Whilst protein quantity can be determined precisely, it is much more difficult to measure protein quality, which is primarily a genotypic trait (Bushuk, 1985).

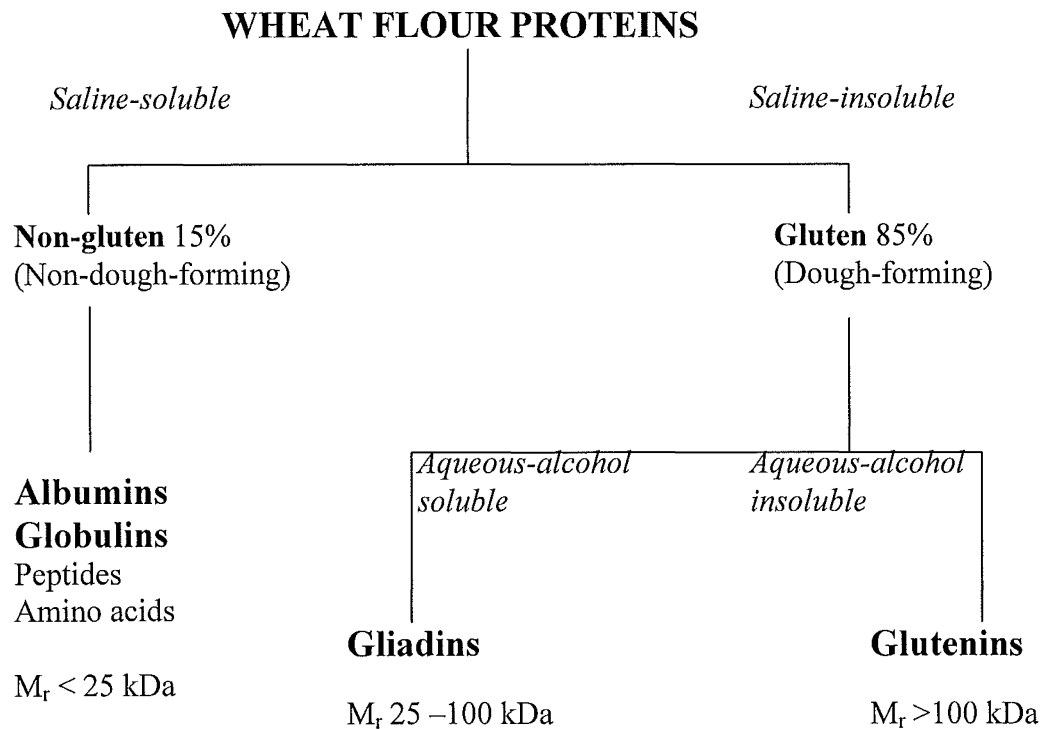
1.2.2 The proteins of wheat flour

Extensive studies of wheat proteins over many years have highlighted their great complexity (Tatham et al, 1990; Shewry et al, 1992; Abdel-Aal et al, 1996; DuPont et al, 2000). These proteins are generally classified according to their solubility differences by the system of Osborne (1907). As shown in figure 1.2, the gliadins and glutenins constitute the so-called gluten fraction of wheat proteins. In the wheat grain, these two groups are the storage proteins, serving as the nutritional source for the growing embryo (Shewry et al, 1995).

Albumins are proteins that are soluble in water and low salt concentrations, while globulins are insoluble in pure water but soluble in dilute salt solutions (Autran, 1993). Albumins and globulins are often classified together as the salt-soluble or “non-gluten” fraction and comprise about 15% of flour protein (Bushuk, 1985). This group of small (molecular weight < 25 kDa) monomeric proteins consists of an enormous number of metabolically active proteins, such as enzymes, regulatory proteins and transport proteins (Lasztity, 1984). Although this fraction is not generally considered to be dough-forming (Frazier, 1992) it has been shown that dough without the water-soluble fraction has different properties from the original dough (Yoshida & Danno, 1989). Nutritionally, the albumins and globulins have a good amino acid balance, with a

higher level of essential amino acids such as lysine than the other protein groups (Autran, 1993).

Figure 1.2: Conventional classification of wheat flour proteins, based on solubility. The salt-soluble fraction consists of small proteins, while the salt-insoluble “gluten fraction” consists of large, monomeric and polymeric proteins.



Gliadins are soluble in aqueous alcohols (typically 60-70% ethanol) and are present as monomeric proteins that either lack disulfide bonds or have only intra-chain disulfide bonds (Shewry and Tatham, 1997). This is due to the absence of cysteine residues in some gliadins and the presence of only intra-molecular cysteine residues in others (Muller & Wieser, 1995). Gliadins are divided into α -, β -, γ - and ω -gliadins, based on their mobility on acidic buffer PAGE (Wrigley & Bietz, 1988). The molecular weight range of the gliadin fraction ranges from around 11-80 kDa when run under reducing conditions on SDS-PAGE (Mimouni et al, 1998). The role of the gliadins in dough development is thought to be one of promoting extensibility and viscous flow, which refers to the ability of the dough to be stretched. This has been demonstrated by

various studies including that of Fido et al (1997), in which the addition of some gliadin fractions to wheat flours increased the extensibility in a quantitative fashion.

Central to the unique properties of wheat gluten are the glutenins, which are the large, polymeric proteins of wheat flour (Wrigley, 1996). Glutenins make up approximately 40% of flour proteins and are mainly responsible for dough elasticity, or 'springiness' and dough strength (Shewry et al, 1995; Gianibelli et al, 2001). The polymers of glutenin cover a wide array of sizes, ranging up into molecular weights of tens of millions, thus gaining the reputation for being among the largest proteins in nature (Wrigley, 1996; Wrigley & Bekes 1999). Whereas gliadins are single-chained, the glutenins are multi-chained and are stabilised by inter-chain disulfide bonds (Autran, 1993). The polymeric glutenins have been reported by Southan & MacRitchie (1999) to consist of A subunits (80–120 kDa), B subunits (40–55 kDa) and C subunits (30–40 kDa) when reduced (refer figure 1.4), forming polymers ranging from 100 kDa to the order of millions. Thus the traditional solubility groups can now be described on the basis of molecular weight distribution, which is determined by the relative amounts of monomeric and polymeric proteins (Southan & MacRitchie, 1999).

However, it is important to note that although some overlap occurs between the albumins and globulins, gliadins and glutenins in terms of size, each protein type has molecular weight, aggregation and sequence characteristics distinct from those of the other groups (Wrigley and Bietz, 1988).

1.2.3 Relationship of structure to wheat protein functionality

The characterisation of wheat flour proteins has been achieved using many techniques including those of biochemistry (Veraverbeke et al, 2000), molecular biology (Kasarda, 1999) and immunology (Mills et al, 2000). They have also been more recently studied in relation to several principles unique to polymers, including segmental motion and molecular weight distribution (Singh and MacRitchie, 2001). As a result, current knowledge of the relationship between protein structure and function is better in this group of food proteins than in many others, but is still incomplete.

Among the known facts about the gluten proteins is the genetic basis for their composition in a given flour variety (Pritchard and Brock, 1994; Wrigley, 2002). It has

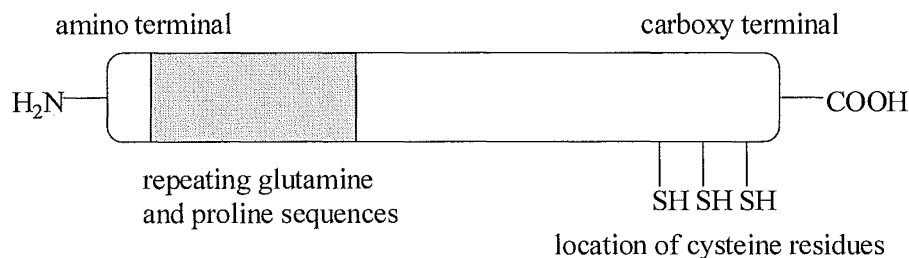
become clear that variations in the alleles that specify the high molecular weight glutenins, in particular, are correlated to bread making quality (Payne, 1987; Nieto-Taladriz et al, 1998; Tatham, 2000). This means that the presence of specific alleles specifying the expression of particular high molecular weight glutenins is an indication of the strength and quality of the flour (Weegels et al, 1996; Shewry et al, 1995).

Thus, in baked products, functional properties are the direct result of flour protein composition (Bushuk, 1985; MacRitchie, 1999). Consistent with proteins in many other foods, it is the structure of these proteins that is ultimately responsible for functionality (MacRitchie and Lafiandra, 1997; Lindsay and Skerritt, 1999). As such, the structural features of the gliadins and glutenins that are thought to contribute to particular properties, will now be considered.

Relationship of gliadin structure to functionality in dough

Gliadins are compact, tightly folded molecules that are unusually stable to thermal treatments (Lasztity, 1984). At least some of this stability is derived from the presence of intra-molecular disulfide bonds. Figure 1.3 shows a schematic representation of a typical gliadin amino acid sequence. The cysteine residues are located at only the carboxy terminal, facilitating the formation of disulfide bonds that are contained within the individual polypeptide chains due to their close proximity.

Figure 1.3: Schematic representation of gliadin sequences. Adapted from Tatham (2000). The amino terminal has a repeating sequence of glutamine and proline residues, whereas the carboxy terminal is non-repetitive and contains all of the cysteine residues, shown by their SH (thiol, also known as sulfhydryl) groups.

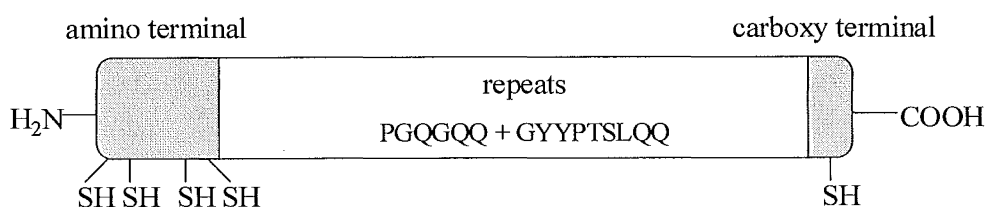


Gliadins have a short region of repeating glutamine and proline sequences at the amino terminal and a non-repetitive carboxy terminal. Studies with purified ω -gliadins, using the techniques of MALDI-TOF mass spectrometry and circular dichroism spectroscopy have shown flexible, coiled structures (DuPont et al, 2000), which have been proposed to play a significant role in the cohesiveness of dough.

Relationship of glutenin structure to functionality in dough

The formation of the gluten network upon hydration of flour depends on the association of the glutenins with one another through both hydrogen (H) and disulfide bonding (Bekes et al, 1994; Belton, 1999). When fractionated by SDS-PAGE in the presence of reducing agents, glutenins separate into distinct subunits, the low molecular weight (LMW) and high molecular weight (HMW) subunits. Of these, the HMW subunits are well known for their contribution to dough properties. HPLC analyses (Sutton, 1991) and studies correlating functional characteristics of every member in a sample wheat variety to protein composition (Gupta et al, 1991; Bekes and Gras, 1999) are among the many types of investigations into the relationship between specific HMW subunit alleles and flour functionality.

Figure 1.4: Schematic representation of the sequences of typical high molecular weight glutenin subunits (Tatham, 2000). The x-type subunits (shown) have four thiol groups at the amino terminal, whereas the y-type subunits have five thiol groups at this terminal. Repeating sequences are characteristic of the middle sections. (Abbreviations: P = proline; G = glycine; Q = glutamine; Y = tyrosine; T = threonine; S = serine; L = leucine).



Two immunologically distinct types of HMW subunits, x-type and y-type, are known. Each contains three distinct regions, as shown in figure 1.4. The central region, which

is repetitive, is flanked by non-repetitive amino- and carboxy-terminal regions (Shewry et al, 1992).

The structural basis of dough functionality in these proteins has been attributed to three features. Firstly, the repeating sequences that are rich in glutamine, can form strong H bonds with adjacent subunits and with gliadins. Secondly, the helical folding of the repeat region into β -turns enables the formation of a “molecular spring” (Wrigley and Bekes, 1999; Belton, 1999). Thirdly, and most importantly for dough properties, is the number and distribution of cysteine residues (one at the C-terminal, and four and five at the N-terminal for x- and y-types respectively) that determines the extent of disulfide crosslinking.

The vital role of disulfide bonds in dough structure is demonstrated in studies such as that of Bekes et al (1994). In this study, the addition of the reducing agent dithiothreitol (DTT) to gluten facilitated the reductive cleavage of disulfide bonds and resulted in dramatic dough-weakening effects, such as mixing time and tolerance to over-mixing. Conversely, the same study reported the complete recovery of the original properties upon re-oxidation treatments of the reduced dough with bromate. This led to the conclusion that the presence of intact disulfide bonds was crucial to the three dimensional structure and function of the gluten proteins. Thus altering the covalent linkages between gluten proteins – in this case the disulfide bonding network – yields dramatic changes in the macroscopic properties of a dough. Therefore, the crosslinking of proteins is addressed in the next section, followed by its significance to the wheat proteins.

1.3 Protein crosslinking

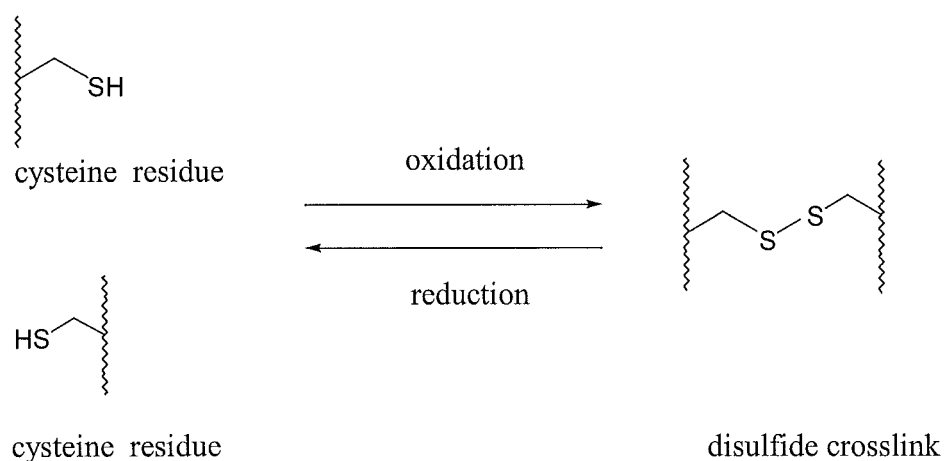
1.3.1 Background

The term “crosslinking” in proteins refers to the covalent bonding of a protein to itself or another protein (Singh, 1991). Crosslinks are widespread in nature, serving to maintain the three-dimensional structure of proteins. They can occur intra-molecularly within the polypeptide chains in a single protein, or inter-molecularly between the same or different proteins. Naturally occurring crosslinks are common in many protein

derivatives, often conferring a degree of rigidity that is lacking in the non-crosslinked forms (Freedman, 1979; Feeney & Whitaker, 1988). The extent of crosslinking in proteins affects their chemical, physical and functional properties (Panyam and Kilara, 1996).

The most thoroughly studied of naturally occurring crosslinks are the disulfide bonds, which exist between cysteine residues in proteins. These bonds, which occur in the keratin of hair and feathers for example, are formed by the oxidative coupling of the thiol groups (figure 1.5), providing stability and rendering the protein resistant to proteolysis (Singh, 1991). Other naturally occurring crosslinks include the aldol and aldime crosslinks that confer strength to mechanical proteins such as collagen and elastin (Folk and Finlayson, 1977). The appropriate degree of crosslinkage is critical for maintaining the correct degree of firmness or elasticity for a particular cell, tissue or organ (Feeney and Whitaker, 1988). Crosslinking also plays a direct role in many important processes such as blood clotting, by means of the transglutaminase-catalysed ϵ -(γ -glutamyl)lysine crosslink (Folk and Finlayson, 1977). Changes to the crosslinking pattern often result in significant alterations to the function of a protein. Such changes are implicated in complex biological processes including diabetes, Alzheimer's disease and conditions associated with ageing (Brownlee, 1994; Singh et al, 2001).

Figure 1.5: Reversible formation of disulfide crosslinks by the oxidation of SH groups in food proteins. (Adapted from Winkler, 1992).

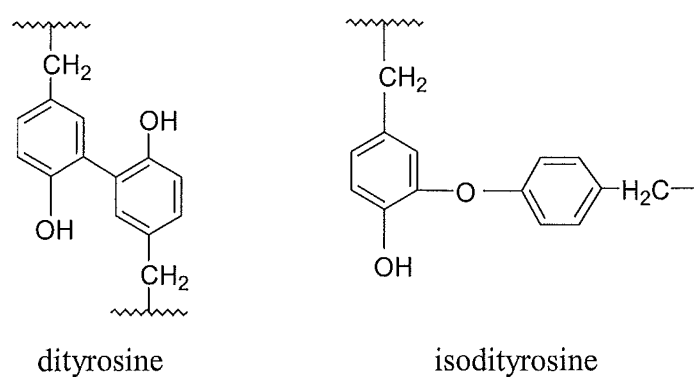


1.3.2 The natural occurrence of crosslinking in wheat proteins

While the occurrence of disulfide bonding has been the focus of much attention for its assumed role in the gluten network, recent work by Tilley et al (2001) has suggested other, non-disulfide type crosslinks, involving tyrosine in particular, to be as important in dough structure and function. Indeed, these researchers have challenged the long-held view of the role of the thiol-disulfide bond interchange, stressing that due to a lack of definitive confirmatory data, it remains a hypothesis. Instead, they suggest that tyrosine crosslinks, in the form of dityrosine or isodityrosine (figure 1.6) are as likely to occur, given their common occurrence in plant proteins and the fact that they are prevalent in extensin (a cell wall glycoprotein) which has amino acid compositions and secondary structures that closely resemble those of glutenin proteins.

These researchers also question evidence that has previously been considered as support for the role of disulfide bonds in dough. With reference to cysteine and glutathione, which have been thought to disrupt the gluten network solely by breaking disulfide bonds, they point out that these are also capable of inhibiting the formation of dityrosine bonds.

Figure 1.6: Structures of dityrosine and isodityrosine.



While it is apparent that these ideas may have a valid experimental basis, they do not explain many of the reversible attributes of dough mixing. The rupture and formation of disulfide bonds and hydrogen bonds have low energy requirements and can easily occur at food processing temperatures; in contrast, tyrosine crosslinks have much higher bond strengths and are less likely to rupture in a reversible process. However,

the demonstration of non-disulfide crosslinks in dough proteins suggests that other methods of crosslinking might be employed to manipulate the overall structure, and thus the function, of the gluten network.

1.3.3 Crosslinking in food proteins

Conventionally, processing technologies are employed for various purposes, including preservation of foods, isolation of food fractions and improvement of the functional properties of foods. Many processing operations, for example mixing, cleaning and pasteurisation, have little or no effect on the nutritional properties of food (Fellows, 2000). However, some conditions have the opposite effect, resulting in undesirable textural changes, or in the deterioration of nutritional and functional properties. One important type of change that can occur is the formation of covalent crosslinks.

In food proteins, crosslinking can have a profound effect on nutritional and textural properties (Motoki and Kumazawa, 2000; Kuraishi et al, 2001). Food processes involving heat treatment, extremes of pH (particularly alkaline), or exposure to oxidising agents frequently lead to substantial changes in protein structure (Singh, 1991; Friedman, 1999), including the formation of intra- and inter-molecular crosslinks, which give the food properties quite different from those of the original proteins.

Another effect that can take place during processing and storage, is the complex set of reactions between sugars and amino groups in proteins, collectively known as the Maillard reaction. This typically results in the formation of many reaction products, leading to the reduction of nutritional quality through crosslinking and the loss of available lysine (Fayle and Gerrard, 2002).

While the onset of crosslinking during processing and storage has generally been considered detrimental to product quality, crosslinks can also be artificially introduced into food proteins chemically or enzymatically, in order to improve functional properties. Enzymes are perceived by consumers to be a more natural way to crosslink food proteins, including those of wheat (Poutanen, 1997; Ferjancic-Biagini et al, 1998). This is mainly because enzymatic modifications, including crosslinking, are specific, require milder conditions than chemicals and are unlikely to lead to the

formation of toxic products (Gerrard et al, 1995; Chobert et al, 1996). In addition, enzymes are used only in catalytic amounts.

1.4 Introduction of crosslinks into food proteins

The deliberate introduction of covalent crosslinks into food proteins can improve functional properties such as texture, product volume, viscosity, emulsification and gelling properties (Hamada, 1992 b). In this section, some chemical and enzymatic methods of introducing disulfide and non-disulfide crosslinks into food proteins are considered, with a particular focus on the wheat proteins.

1.4.1 Chemical disulfide crosslinking

As discussed above, disulfide crosslinks are formed by the oxidative coupling of two thiol groups from cysteine residues (figure 1.5) within the same protein or between adjacent ones. Oxidising and reducing agents affect the mechanical properties of gluten or dough very strongly by modifying the redox status of the glutenin subunits. Chemical crosslinking agents such as potassium bromate are assumed to act by the oxidation of thiol groups, to disulfide bonds (Allen, 1999).

The scheme shown in figure 1.5 is also applicable to ascorbic acid, a commonly used flour improver, which is thought to exert its effect through its first stable oxidation product, dehydro-L-ascorbic acid (DHA). When dough is mixed, ascorbic acid is oxidised by atmospheric oxygen to DHA, which then oxidises the thiol groups of cysteine residues, forming disulfide bonds. The increase in the number of disulfide bonds is thought to result in a strengthening of the gluten network, which is reflected in improved functional properties in baked products. The oxidation of ascorbic acid to DHA and the accompanying conversion of thiol groups in gluten proteins to disulfide bonds is discussed in more detail in section 1.5.

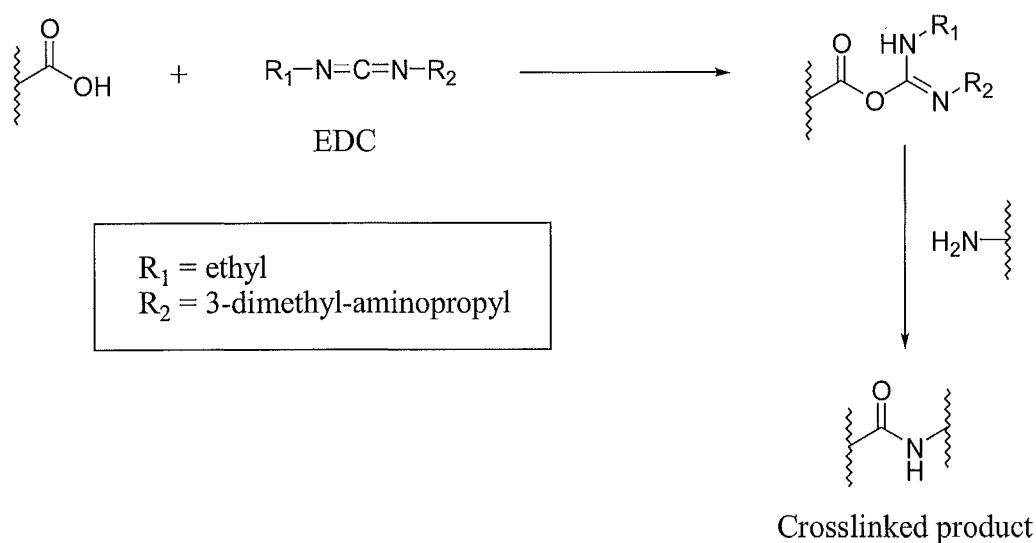
1.4.2 Chemical non-disulfide crosslinking

Non-disulfide, as well as disulfide crosslinks can be introduced into food proteins, by chemical means. Crosslinking chemicals are usually bifunctional compounds with two reactive groups, and can be employed to add intra- or inter-molecular crosslinks into

proteins including those of foods (Singh, 1991). For example, dialdehydes react with the amine groups of lysine residues, the phenol groups of tyrosine or the sulfhydryl groups of cysteine residues (Tropini et al, 2000). Many such crosslinking agents are available commercially (Pierce, 2001) including those designed specifically for site-directed conjugation of proteins, as well as the more traditional reagents such as glutaraldehyde.

Reagents such as these are expensive and it is difficult to use them to add crosslinks in a manner approved for food. For example, glutaraldehyde, a highly reactive reagent with a relatively simple structure, can form crosslinks between proteins through a wide range of reaction mechanisms (Avrameas, 1969). Although these compounds are not used for the crosslinking of food proteins for consumption, they have been studied in our laboratory for the purpose of understanding the crosslinking of food proteins by Maillard-type chemistry (Gerrard et al, 2002 a, b, c).

Figure 1.7: Crosslinking of proteins with EDC as illustrated by Tropini et al (2000).

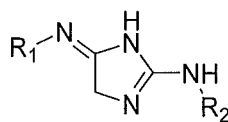


Other chemicals which have been used for the crosslinking of proteins from food include the water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC) (Tropini et al, 2000), which has been used to polymerise wheat gluten *in vitro*. This involved the activation of carboxylic acid groups in the gluten proteins by EDC, followed by their reaction with free amino groups of the same

or another polypeptide chain, resulting in intra- or inter-molecular crosslinks (figure 1.7). The purpose for their work was to improve the functional properties of wheat gluten for non-food uses such as adhesives, coatings and thermoplastic materials. They found that the extent of crosslinking could be controlled with the reaction conditions, particularly pH, depending on the application required.

Protein crosslinks resulting from the Maillard reaction during food processing can also significantly affect functional properties (Mohammed et al, 2000). It has been recently shown that lysine-arginine crosslinks formed during food processing play a major role in food protein polymerisation (Biemel et al, 2001). These crosslinks can be formed from the reaction of proteins with dicarbonyl compounds such as glyoxal, a breakdown product of glucose. An example of such a crosslinked compound is 2-ammonio-6-(2-[(4-aminio-5-oxido-5-oxopentyl)amino]-4,5-dihydro-1H-imidazol-5-ylidene)amino) hexanoate (GODIC). The structure of this compound is shown in figure 1.8.

Figure 1.8: Structure of the crosslinked compound GODIC (Biemel et al, 2001).



GODIC

1.4.3 Enzymatic disulfide crosslinking

Due to the significance of disulfide bonds in food, enzymes that add these crosslinks to proteins have been of interest in the food industry. In particular, protein disulfide isomerase (PDI) has been noted for its direct action on gluten proteins (Hillson et al, 1984; Bulleid and Freedman, 1988; Watanabe et al, 1998). Others, such as glucose oxidase (Vemulapalli et al, 1998) and hexose oxidase (Poulsen and Hostrup, 1998) are thought to exert their oxidative effect indirectly due to the production of hydrogen peroxide. The potential use of enzymes such as PDI to catalyse disulfide bond formation in gluten proteins has been reviewed by Shewry and Tatham (1997). The effects of these enzymes on dough are described in more detail in sections 1.5.2.

1.4.4 Enzymatic non-disulfide crosslinking

Of all the enzymes that can be used in baking as crosslinking agents, transglutaminase may be the most suitable, due to its mechanism, which is independent of the complex redox chemistry present in dough. The addition of non-disulfide crosslinks to food proteins by this enzyme has been the subject of much attention (Motoki and Seguro, 1998; Zhu et al, 1995; Kuraishi et al, 2001).

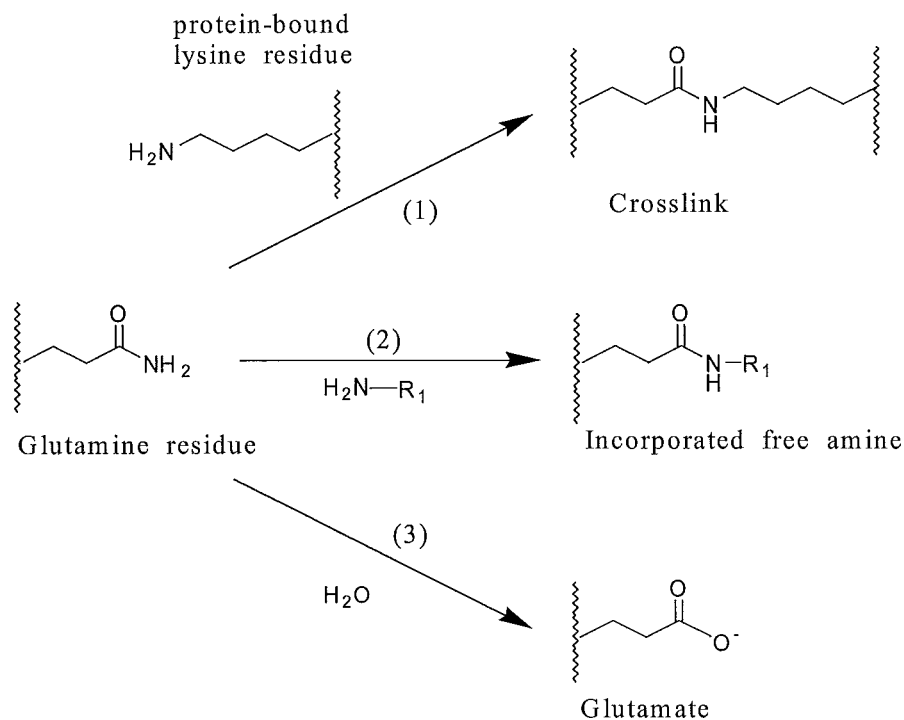
Throughout the food industry, the transglutaminase group of enzymes (protein-glutamine γ -glutamyl transferase, EC 2.3.2.13, abbreviated to TGA) has been acclaimed as an excellent potential means of modifying the properties of food proteins (Kuraishi et al, 2001). An ever-increasing number of reports describe the favourable results obtained when this enzyme is employed for the processing of such products as fish (Zhu et al, 1995), meat (Ando et al, 1989; Kuraishi et al, 1997) and dairy products (Nonaka et al, 1992; Faergemand et al, 1997). Very few reports, however, focus on using TGA in wheat-based, baked goods (Rohm et al, 1995; Gerrard et al, 1998, 2000; Poza, 2002). Most notable of these are the reports of Gerrard et al (1998, 2000) investigating the addition of TGA to doughs of bread and laminated pastries such as croissants and the resulting improvements to functional properties. These are described in section 1.5.3.

TGA catalyses the acyl transfer reaction between the γ -carboxamine group of a peptide-bound glutamine residue and the primary amino groups of various substrates, including lysine residues (Nonaka et al, 1989). This reaction results in the formation of irreversible intra- or intermolecular ϵ -(γ -glutamyl)lysine crosslinks (figure 1.9, reaction (1)). The formation of these crosslinks occurs in the presence of amine substrates. Without amine groups in the medium, water becomes the acyl acceptor, the catalysed reaction then becoming the deamidation of glutamyl residues (figure 1.9, reaction (3)). This results in improved solubility of the proteins (Alexandre et al, 1993). A third reaction that is catalysed by TGA is the incorporation of amines into proteins (Nonaka et al, 1996, figure 1.9, reaction (2)), including the attachments of chemical labels or probes (Ikura et al, 1981). This reaction has been utilised to increase digestibility of wheat gliadin (Iwami and Yasumoto, 1986).

TGAs are found in many tissues and body fluids of vertebrates and are known to be involved in many biological processes including blood clotting (plasma factor XIII) and wound healing (Wilhelm et al, 1996). They have also been found in plants (Serafini-Fracassini et al, 1995) and micro-organisms such as *Streptovercillium* spp. (Ando et al, 1989; Ho et al, 2000).

The use of TGA in foods has greatly increased since its purification and mass production from micro-organisms by fermentation and cloning technologies (Washizu et al, 1994; Gerber et al, 1994, Zhu et al, 1998). The microbial TGA differs from the mammalian enzyme in that it does not require Ca^{2+} for activity. Specific reported improvements to food products include increased firmness and water-holding capacity in frozen dairy products (Nielsen, 1995; Kuraishi et al, 2001), smoother texture in soya products (Motoki and Seguro, 1998), and improved shelf-life in meat (Zhu et al, 1995; Nielsen, 1995).

Figure 1.9: The reactions of transglutaminase. (1) formation of the ϵ -(γ - glutamyl) lysine cross-link; (2) incorporation of free amine into glutamine; (3) glutamic acid formation in the absence of free amines. R_1 represents a wide range of small molecules.



In wheat proteins, the crosslinking effect of TGA has mainly been studied *in vitro*. Such studies have provided much useful information about the nature of the TGA reaction on gluten proteins. Larre et al (1993; 2000) studied the reaction products resulting from incubating gluten with TGA *in vitro* and reported that of the three possible reaction pathways of TGA, the change in gluten behaviour was mainly due to the crosslinking reaction (figure 1.9, reaction (1)). In other *in vitro* studies, the functional properties of gluten have been shown to improve when digestion with proteolytic enzymes such as papain was followed by treatment with TGA (Babiker et al, 1996).

While these studies have provided invaluable information about the effect of TGA on gluten proteins, they are not necessarily entirely reflective of the effect exerted by the enzyme within the food system itself. To this end, research in our laboratory has focussed on the effect of TGA on dough proteins *in situ* (Gerrard et al, 1998 a, b; 2000; 2001). Also, the rheological properties of bug-damaged wheat doughs have been studied and improved when TGA was blended with the flour (Koksel et al, 2000).

The remainder of this review is devoted to the improvement of the functional properties of baked products through the introduction of crosslinks to the component proteins.

1.5 Improvement of wheat dough functionality by covalent crosslinking

The baking industry is one area in which the changes caused by crosslinking are considered desirable (Gerrard, 1998). Many flour varieties are denoted as “weak”, referring to the poor functional properties displayed by their doughs and baked products. Therefore, chemical flour improvers such as potassium bromate (Allen, 1999) and ascorbic acid (Nakamura and Kurata, 1997) have traditionally been employed, to improve the strength and baking performance of such flours.

Bread making relies on the highly cohesive nature of the gluten matrix, which stems from a combination of elasticity and extensibility, or viscous flow (MacRitchie, 1999).

This combination allows the expansion of the dough and entrapment of carbon dioxide during fermentation, leading to a light porous structure that is gelatinised by heating, resulting in the baked loaf (Cornell and Hoveling, 1998).

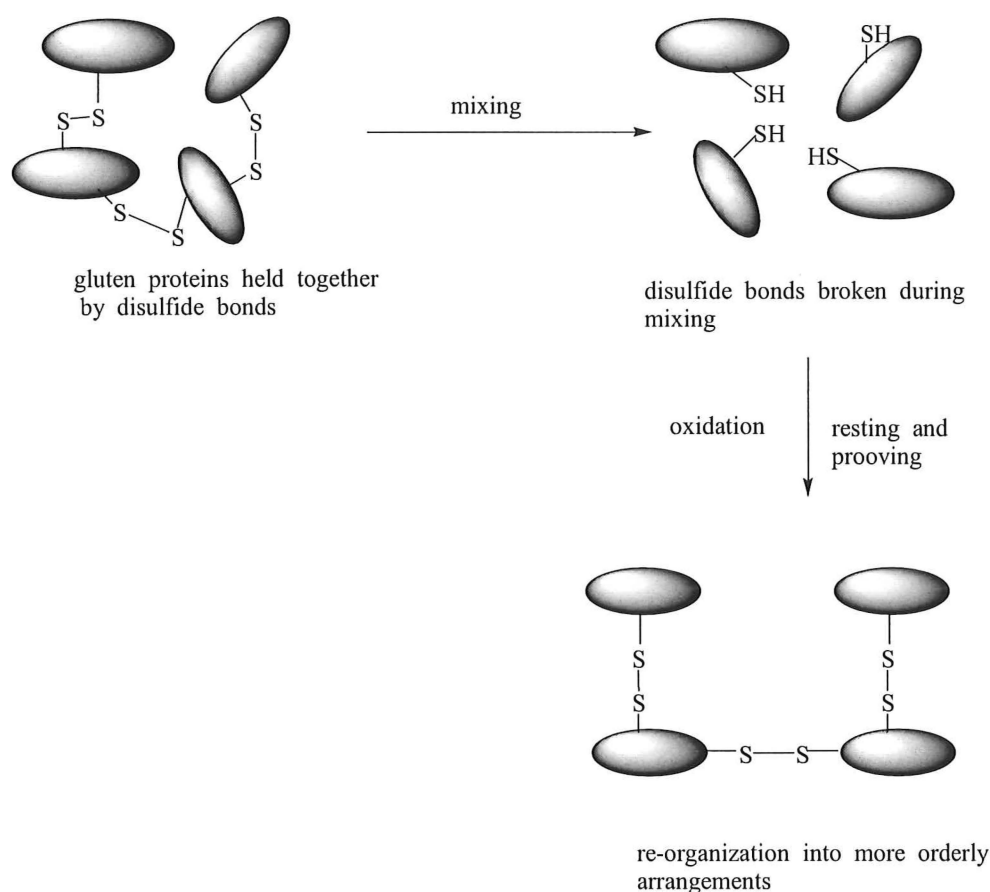
Although both gliadin and glutenin proteins are responsible for viscoelasticity, the strength of the dough mainly lies in the extent of crosslinking that exists between the gluten proteins, particularly the glutenins (Gupta, 1991). Dough strength is a quality parameter that affects several functional properties. It is also a determining factor in the ability of the dough or pastry to withstand freezing (Stauffer, 1993). As described in section 1.2.3, the gluten macropolymer is formed by covalent crosslinking between the cysteine residues of the glutenins as well as H-bonds (Belton, 1999). Thus, the addition of extra crosslinks is likely to facilitate the formation of a stronger network.

In dough, thiol groups can undergo the disulfide-thiol interchange (Dong and Hoseney, 1995; Li and Lee, 1998; Allen, 1999). This is known to significantly affect functionality (Shewry and Tatham, 1997; Anderson and Ng, 2000). The gluten coils, which are held together by disulfide linkages, are unravelled during mixing with the breaking of the disulfide bonds. Breakage can occur not only through the mechanical mixing process but also by the action of naturally occurring enzymes during fermentation (Fischer, 1985). Following fermentation and mixing, it is necessary to reconnect some of the broken links. Oxidative agents introduced into the dough reform the disulfide links, in more orderly alignments, imparting improved strength and elasticity to the gluten structure (Skerritt et al, 1999). This is known as dough development and is illustrated in Figure 1.10. Although reversible, this effect is considered by bakers to be crucial to the production of quality baked goods (Allen, 1999).

Doughs that are under-oxidised are characteristically weak and soft, being difficult to process. Finished loaves from such doughs have a reduced volume, weak crusts and uneven texture and symmetry (Bloksma, 1971). Over-oxidised doughs, on the other hand, display the opposite characteristics, being tight, difficult to mould and easily ruptured. The lack of elasticity in these doughs often results in their breaking open during the proving stage. Breads of over-oxidised doughs are small in volume, with an uneven crust. The gas cells, the size and number of which normally influence crumb

structure, are uneven in over-oxidised doughs, appearing as large holes (Fischer, 1985; Cauvain, 1998).

Figure 1.10: Schematic representation of the breaking of disulfide crosslinks during dough mixing and their subsequent re-formation during proving.



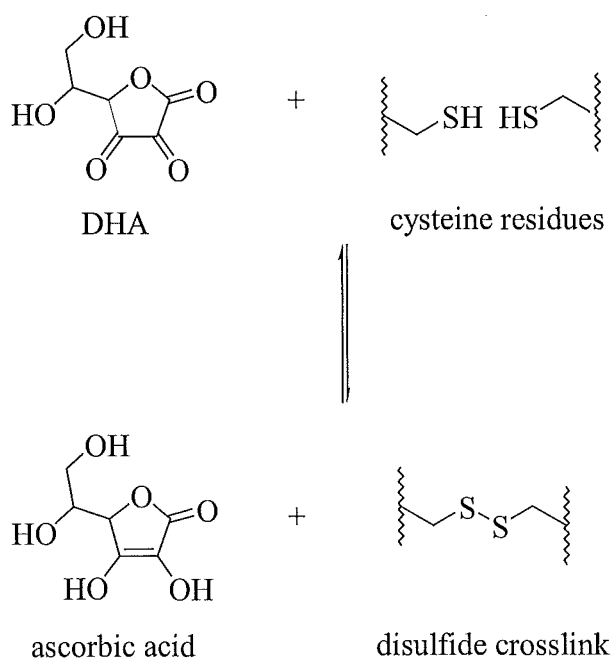
It is important to note that the chemical processes occurring during dough mixing involve more than just the re-organisation of the reactive groups described above. In a complex system such as dough, the thiol-disulfide interchange alone does not provide a mechanism that fully accounts for dough elasticity (Belton, 1999). Hydrogen bonding is also thought to play a major role in the formation of protein "loops" upon hydration of the flour, which in turn contribute to the overall behaviour of the gluten network (Belton, 1999; Callaghan et al, 1999). Whilst the "loop and train" model (Belton, 1999) acknowledges the existence of disulfide bonds within and between protein chains, it suggests they do not necessarily have a major role in dough mixing, but rather have a structural role in the nature of the protein aggregate. This idea has

been supported by the work of other investigators. For example, Wesley et al (1998) used near infrared (NIR) spectroscopy to show the strong involvement of water-mediated hydrogen bonding during dough development. Similarly, Sutton et al (2000) used thiol-labelling techniques to show that disulfide cleavage may not necessarily be a critical process during dough development. Nevertheless, the simple model depicted in figure 1.10 accounts for much of what is observed during the dough development process.

1.5.1 Improving dough functionality with chemicals

In spite of the debate over the relative importance of disulfide crosslinks in dough formation, it remains clear that oxidising chemicals can facilitate improvements to dough functionality. Ascorbic acid (Nakamura and Kurata, 1997) and potassium bromate (Allen, 1999) are among the most well known chemical flour improvers. Ascorbic acid is a reducing agent, thought to improve dough through its first stable oxidation product dehydro-L-ascorbic acid (DHA) (Nakamura and Kurata, 1997). The mixing process introduces atmospheric oxygen into the dough, oxidising ascorbic acid to DHA, which can then oxidise thiol groups in gluten proteins to disulfide crosslinks, as shown in figure 1.11.

Figure 1.11: Oxidation of ascorbic acid to DHA during dough mixing facilitates the formation of disulfide bonds between gluten proteins. Adapted from Winkler (1992).



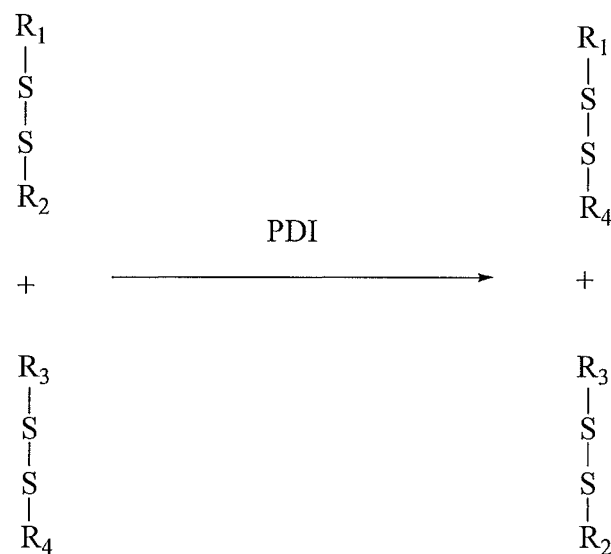
Potassium bromate (KBrO_3) is a slow-acting oxidising agent that works during fermentation, proofing and baking. Its effects on processing, tolerance to mechanical abuse and dosage variations, among other benefits, have long been recognised. However, health concerns were raised based on conflicting scientific evidence, on the potential carcinogenic effects of KBrO_3 (Giesecke and Taillie, 2000). Since then KBrO_3 has been banned for use in bread in many countries and voluntarily removed in New Zealand, thus necessitating the search for an effective replacement. So far, most chemical agents have proven costly, particularly due to the requirements for oxygen associated with their use.

Once again, it must be emphasised that whilst it has been assumed (Shewry and Tatham, 1997; Allen, 1999) that the mechanism for dough strengthening by these chemicals is the formation of disulfide bonds, other crosslinks may also play a role (Callaghan et al, 1999; Tilley et al, 2001; Gerrard et al, 2002 a, b, c). Since the key to the improvements appears to be the increase in the number of crosslinks holding the gluten network together, it seems that the added crosslinks do not necessarily have to be disulfide in nature. Thus oxidation *per se* may not be required to improve dough properties.

Thus chemical methods have been employed to effectively add crosslinks into dough, thereby facilitating certain improvements. The following sections describe the consequences of adding crosslinks into the dough matrix of baked products, by enzymes.

1.5.2 Improving dough functionality by means of the enzymes PDI and glucose oxidase

Several enzymes are able to regulate the disulfide crosslinking reaction, known as the thiol-disulfide interchange. Protein disulfide isomerase (PDI) is one such enzyme used for this purpose in the food industry (Hillson, 1984; Matheis and Whitaker, 1987). The enzyme has been detected in most vertebrate tissues and in extracts of peas, cabbage and yeast (Singh, 1991). PDI has also been investigated in bread (Watanabe et al, 1998), for its effects on dough rheology. The mechanism of action of this enzyme on gluten proteins is outlined in figure 1.12.

Figure 1.12: Reaction catalysed by PDI.

Glucose oxidase is another enzyme which has been used in the baking industry. The oxidative effect of this enzyme is attributed to the hydrogen peroxide produced from oxygen during the catalytic conversion of D-glucose to D-gluconic acid (Miller and Hosney, 1999). Attempts to improve bread dough performance by crosslinking of proteins include the study of Vemulapalli et al (1998), who used glucose oxidase for this purpose. Bread loaves prepared with various doses of glucose oxidase were tested for quality parameters such as crumb strength, loaf volume and water absorption. The loaf volumes of bread with or without the enzyme were not found to be significantly different. The improvements mainly occurred in crumb grain characteristics, such as crumb texture and strength. However, glucose oxidase was not as effective as potassium bromate in improving either crumb grain or loaf volume.

Doughs with added glucose oxidase in the above study were reportedly much drier than controls. It was presumed that hydrogen peroxide, produced by the oxidation of glucose to gluconic acid, was responsible for adding disulfide crosslinks, which caused the observed changes. Similar observations were reported by Miller and Hosney (1999), who also assumed that the strengthening and drying effects were due to the addition of disulfide crosslinks by the hydrogen peroxide, from the conversion of glucose to gluconic acid. In our present research, the crosslinking action of glucose

oxidase was investigated in the proteins of bread and croissants. These studies are described in *Chapter Four* of this thesis.

1.5.3 Improving dough functionality by means of TGA

The largest reported impact to dough properties by a crosslinking agent is that exerted by the enzyme TGA (Gerrard et al, 1997; Gerrard et al, 1998 a; 2000; 2001). These studies have demonstrated remarkable improvements to doughs of bread, croissants and other pastries by the addition of TGA-mediated non-disulfide crosslinks.

Initial work included the comparison of TGA-treated white pan bread with controls for a variety of quality parameters (Gerrard et al, 1998 a). In non-yeasted doughs, dough development was quantified by measuring relaxation time, which refers to the time taken by dough to recover after being compressed by a standard force (Frazier, 1992). A longer relaxation time indicates enhanced dough development. TGA had a profound effect on the bread doughs; relaxation times were increased and surpassed those of standard doughs early in the resting stage. The effect was greater than in doughs treated with the standard improvers ascorbic acid and bromate.

Doughs with added TGA required a lower work input than standard doughs. In the yeasted baked loaves, those with added TGA exhibited improved crumb texture as indicated by bake scores. Crumb strength, a measure of the ability of sliced bread to withstand handling, was also improved. The volume of the baked bread loaves with added TGA was, however, not significantly increased compared to the controls.

Following these observations in bread, studies were extended to other bakery products, particularly croissants and puff pastries (Gerrard et al, 2000). The improving effect of TGA was also tested for preservation during freezing, a process that normally produces deterioration in quality. In pastry, TGA substantially improved pastry lift, resulting in products with increased height (figure 1.13). However, the most dramatic effects were observed on baked croissants, where volume was greatly increased by the addition of TGA (figure 1.14). This effect was observed in croissants baked both with white flour and a 50:50 blend of wholemeal and white flours. Furthermore, these effects on croissants and pastries were maintained in frozen storage for up to three months.

Figure 1.13: Effect of the addition of TGA on pastry (Gerrard et al, 1997).

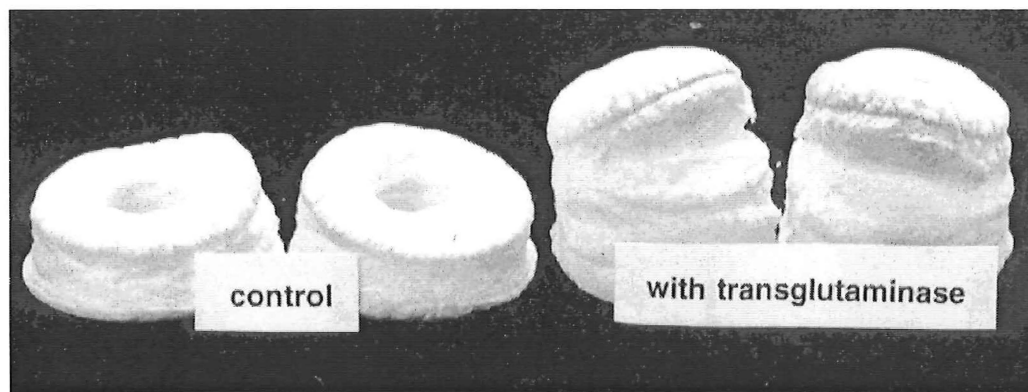
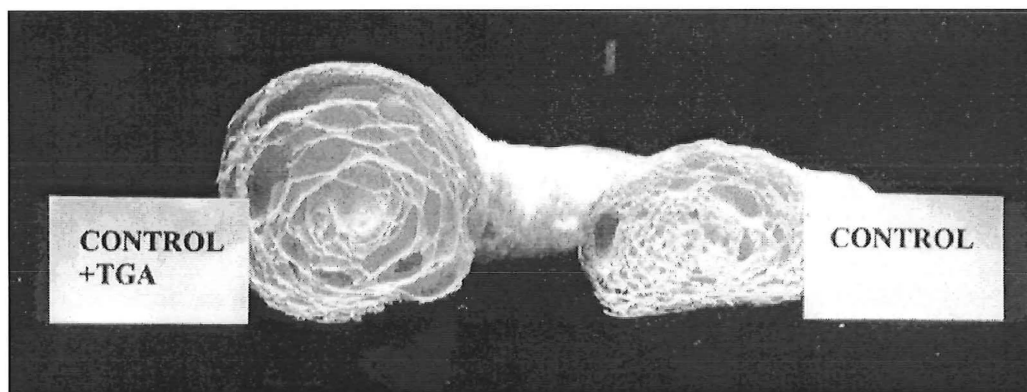


Figure 1.14: Effect of the addition of TGA to croissants (Gerrard et al, 1997).



The difference in product volume between bread and croissants is thought to be related to processing factors (Gerrard et al, 2001). Lamination in croissant preparation results in a layered dough in which the effect of TGA on each dough layer gives an apparently exaggerated effect, whereas in bread the effect is on the dough as a whole. Crosslinking of the proteins by TGA may thus strengthen the individual pastry sheets, giving the croissant better gas-retaining properties. In bread, strengthening of the dough prevents expansion which results in reduced loaf volume. Thus strength, rather than elasticity of the dough, appears to be improved by TGA.

In view of the promising results obtained with baked products, the present work was initiated to further investigate the improvement of baked products by enzymatic protein crosslinking. TGA and glucose oxidase were researched and compared for their different mechanisms of adding crosslinks to wheat proteins. These were then related to differences in the resulting changes to baked products.

1.5.4 Further possible advantages of TGA in wheat flour products

While the obvious improvements described above represent the main reasons for the use of TGA in baked goods, additional advantages could also result from the treatment of flour with this enzyme. One limitation of the wheat proteins that may be addressed in this context, is their insolubility. The deamidation of proteins is known to increase their solubility (Friedli & Howell, 1996). The deamidation reaction, which is the third reaction of TGA, takes place if there is insufficient amine to facilitate the crosslink formation reaction (Motoki & Seguro, 1998). If this is the case, water becomes the acyl acceptor, causing the number of carboxylic acid groups to increase, thereby increasing the overall solubility and thus the digestibility of the protein. Hence the use of TGA may increase the digestibility of wheat proteins.

A further advantage of TGA relates to the essential amino acid lysine, which is limiting in wheat flour and in cereal proteins in general. It has been shown that the lysine residues are protected within the crosslink and are available nutritionally (Seguro et al, 1996) and that although irreversible, the ϵ -(γ - glutamyl) lysine crosslink is completely digestible in the gut (Kurth & Rogers, 1984). This is significant in terms of the improvement of the nutritive value of flour, particularly since processing and

storage conditions often further reduce lysine availability. It is also possible to mediate the incorporation of limiting amino acids into proteins through TGA treatment, as described by Ikura et al (1981) and Nonaka et al (1996). The former researchers used mammalian TGA to incorporate lysine into wheat gluten, while the latter employed microbial TGA to incorporate lysine and lysine dipeptides into casein. In addition, Iwami et al (1986) have demonstrated the digestibility of lysine attached in this way, to wheat gliadin. The protection of lysine as a result of TGA treatment is investigated in *Chapter Five* of the present research. Lysine measurements were taken on protein extracts from bread and croissants, both to investigate the effect of TGA on lysine and as a means of monitoring the reactivity of TGA.

The coeliac response constitutes the final incentive for the application of TGA to flour products. Several investigations, including those of van der Wal et al (1998) and Watanabe et al (1994) have indicated the possibility of reducing the allergenicity of wheat proteins with TGA treatment.

1.6 Thesis overview

This thesis presents results from investigations of specific changes to dough proteins caused by food enzymes and the corresponding changes to their functionality in baked products.

In *Chapter Two*, the changes brought about by TGA are examined at the molecular level, using the techniques of SDS-PAGE and HPLC, and explanations at the molecular level suggested for the observed product improvements.

Due to the increase in worldwide production of frozen doughs, *Chapter Three* investigates the use of TGA for quality preservation in frozen croissant doughs. The maintenance of the results of crosslinking over three months in a -20°C freezer are studied.

The effects of the second crosslinking enzyme, glucose oxidase, are explored in *Chapter Four*. Once again, while overall changes to product quality are reported, the

main emphasis is placed on the molecular modifications responsible for the observed effects.

Chapter Five reviews ways of measuring TGA activity and compares the measurement of lysine content and the hydroxamate assay as assay methods. A new method for measurement of TGA activity is developed, using an image analysis protein quantification technique, initially in three model proteins and then in a HMW subunit of glutenin.

Chapter Six discusses the research as a whole, comparing the enzymatic crosslinking of wheat proteins brought about through non-disulfide and disulfide means. The introduction of non-disulfide protein crosslinks by the TGA reaction is put forward as an excellent tool for the improvement of dough properties, making this enzyme an ideal processing aid for the baking industry.

Finally, in *Chapter Seven*, the materials and experimental procedures used for the testing of the above hypotheses are described.

1.7 References

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Chapter Two

TRANSGLUTAMINASE – INDUCED MOLECULAR CHANGES TO DOUGH PROTEINS

2.1 Introduction

The dramatic effects caused by the addition of transglutaminase (TGA) to doughs of bread and other baked products were described in *Chapter One*. The research described in this chapter has been published in the Journal of Food Science (Gerrard et al, 2001), and relates the observed improvements in product functionality to structural changes in dough proteins effected by TGA.

In bread, the functional properties reported to be improved by TGA, included increased dough relaxation time, increased water absorption and lowered work input. Since a longer relaxation time indicates a stronger dough, this suggested that TGA could improve the quality of weaker flours. It also implied that dough development, usually aided by chemical oxidising agents thought to act *via* the addition of extra disulfide crosslinks, may be enhanced instead through the non-disulfide crosslinking mechanism of TGA (Gerrard et al, 1998 a). A lowered work input during the dough mixing process has cost-saving benefits, while a higher water absorbing capacity signifies a less extensible, stronger dough (Larsen and Greenwood, 1991). Other reported benefits of adding TGA included improvements in crumb strength and texture.

In layered pastries, such as croissants, TGA markedly increased product volume. TGA-treated croissant doughs were stiffer and less extensible than controls (Gerrard et al, 2000). It was also found that visible damage occurred to control samples with the leakage of butterfat on proofing, whereas no such effect took place in TGA-treated samples. Puff pastries and croissants baked with TGA had a much better texture,

mouthfeel, layer and step structure than the controls. Furthermore, these improvements were preserved upon freezing for up to three months (Gerrard et al, 2000).

In light of these findings, and the increasing use of TGA as a processing aid throughout the food industry, this research set out to determine the exact nature of the changes incurred to the proteins of wheat dough. This was necessary in order to understand the action of the enzyme and relate changes caused at the molecular level, to the macroscopic changes in the functional properties of baked products. While it has generally been assumed that the improvements are the result of the crosslinking activity of TGA on food proteins, direct experimental evidence for this supposition is limited, particularly in wheat-based foods. It was also considered important for the studies to be conducted on the doughs *in situ*, as results obtained with proteins in buffer systems may not necessarily reflect changes that occur during food processing.

2.2 The role of proteins in dough

2.2.1 Dough formation

It is the protein component of wheat flour that is chiefly responsible for the properties that make it suitable for leavened products (Bushuk, 2000). When wheat flour is mixed with water, a viscoelastic mass called gluten is formed by the extrusion of protein microfibrils (Bernadin and Kasarda, 1973). These strands are stretched and orientated during mixing. An optimum level of mixing gives rise to the gluten network, the strength and elasticity of which determine the functional properties of the baked product, including volume and gas-retention (Bushuk, 2000; Graveland et al, 2000).

Wheat gluten is a unique material because it can be stretched, fermented and gelatinised by heating, giving rise to a great variety of baked products including breads, pastas, noodles, pastries and biscuits. Wheat gluten can also be separated from flour and used for a variety of purposes, particularly as an additive to low protein flours to improve their breadmaking performance (Magnuson, 1985). Thus, manipulating the properties of gluten offers a means to improve the quality of wheat-based products.

Several chemical bonds, both covalent and non-covalent, are important in dough. It has been reported that, in order of increasing strength, the four main types are: hydrophobic forces, hydrogen bonds, electrostatic interactions and covalent interactions (Dickinson, 1997). These chemical bonds must ultimately be responsible for the macroscopic properties of the dough and final baked product. Manipulation of these bonds, particularly covalent interactions, therefore offers a means by which to manipulate the properties of baked produce. Before this manipulation can proceed in an informed manner, we need to understand how particular bonds relate to the macroscopic properties of a dough. Various parameters have been adopted to quantify such macroscopic properties, amongst them dough development.

As described in *Chapter One*, dough development in the breadmaking process refers to the breakage of existing disulfide bridges during mixing and their subsequent reassembly during proofing, into more orderly arrangements (see figure 1.10). The dramatic changes to dough upon addition of TGA required an explanation at the molecular level, in order to understand the full action of TGA on wheat proteins.

2.2.2 Measuring changes in wheat flour proteins

In order to determine specific molecular changes induced by the addition of TGA to wheat-based foods, the four protein fractions described in sections 1.2.2 and 1.2.3 were routinely extracted from doughs of bread, croissants and biscuits. Extraction was carried out using the method of Hay and Sutton (1990), based on the Osborne (1907) fractionation technique. In the case of croissants and biscuits, the fat was first removed from doughs by the Soxhlet method prior to protein extraction. Albumins and globulins were extracted in a dilute salt solution, while gliadins were extracted in 70% ethanol. The glutenins were extracted into two groups, those soluble in an SDS-phosphate buffer (SDS-soluble glutenins) and those that could only be solubilised by the use of sonication (SDS-insoluble glutenins).

2.3 Choice of methods for the characterisation of TGA-induced changes to wheat proteins

For many reasons including their heterogeneity, wheat storage proteins are generally considered difficult to work with. Due to their poor solubility and the complex structures and interactions within the polymeric network, considerable innovation has been required to modify traditional techniques of electrophoresis and chromatography for the characterisation of these proteins (Bietz and Kruger, 1994).

In order to relate improvements in functional properties to specific molecular changes, the different protein groups were extracted from control and TGA-treated doughs and compared by SDS-PAGE and HPLC. To observe the results of crosslinking by SDS-PAGE, this work employed methodology similar to that of Aboumahmoud and Savello (1990) in their study of the TGA-mediated crosslinking of whey proteins. The extent of crosslinking of the whey proteins was demonstrated by the disappearance of certain protein bands, the appearance of new ones and the accumulation of immobile protein polymers at the gel origin. In this way, changes to the proteins due to intermolecular crosslinking could be readily detected. In our work, occurrence of TGA activity was evidenced in an analogous manner.

HPLC has also been used to monitor the crosslinking of food proteins by TGA. In the study of Motoki and Nio (1983), the increasing formation of polymers as a result of crosslinking was reflected in an increase of the peak at void volume (at the beginning of the run, corresponding to the top of the resolving gel on SDS-PAGE) where molecules too large to enter the column support are eluted.

Since both SDS-PAGE and HPLC have been extensively employed in the analysis of separated wheat fractions, it was decided to use both methods for the characterisation of TGA-induced changes to wheat proteins. In our investigation, SDS-PAGE and HPLC provided consistent and complementary information on the protein fractions of control and TGA-treated doughs of bread, croissants and biscuits. SDS-PAGE proved to be the method of choice for establishing qualitative changes to protein fractions, whereas HPLC permitted quantitation of the specific changes to protein fractions. Both

methods were able to show changes with a small sample size and proved suitable for the efficient analysis of multiple samples. Furthermore, the same extracts could be analysed by both techniques, eliminating the requirement for separate extraction procedures.

2.3.1 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is widely used for the separation of proteins on the basis of their relative mobilities, which are dependent on the molecular weight of the component polypeptides (Hames, 1981). Due to its applicability over a wide size range, SDS-PAGE is useful for the separation of wheat proteins and, as such, has been employed in many investigations over several years (Moonen et al, 1982; Fu and Sapirstein, 1996; Gianibelli et al, 2001) since its introduction to cereal chemistry by Bietz and Wall (1972).

SDS-PAGE of wheat proteins has been carried out in order to characterise the sizes of different wheat proteins (Mimouni, 1998), to determine genetic relationships between varieties (Khan et al, 1985) and to evaluate the contributions of specific proteins to bread-making quality (Huang and Khan, 1997a, b, c). It has also been conducted in conjunction with other methods, in particular HPLC (Sutton, 1991; Gupta et al, 1996).

SDS-PAGE was thus very suitable as a routine procedure in this investigation, particularly in studying changes to protein size caused by inter-molecular crosslinking. An important initial consideration was determination of the most suitable concentration of acrylamide for these studies. Wheat protein studies have used several acrylamide concentrations depending on the particular pore sizes required. In addition to gels of uniform concentrations, gradient gels have been employed, the increasing acrylamide concentration slowing the proteins as they migrate thereby sharpening the resulting bands (Cornell and Hoveling, 1998). In our research, uniform concentrations of 7 or 12% acrylamide were initially tested. While both concentrations proved suitable for the analysis of dough proteins, the 7% concentration proved more effective in allowing some of the very large polymers resulting from TGA activity to enter the gels. As such, this concentration was employed in this part of the research as the standard electrophoretic protocol.

All samples were treated with the reducing agent mercaptoethanol prior to SDS-PAGE, thereby disrupting the naturally occurring disulfide bonds (as described by Cornell and Hoveling, 1998). This was to ensure that any changes were due solely to non-disulfide crosslinks mediated by TGA. This was confirmed in initial experiments in which samples that were run without prior reduction produced streaked lanes showing higher molecular weight material than their reduced counterparts, but with no clearly identifiable bands. Reduction of the samples also assisted the entry of the proteins into the gels. Although the breaking of the native disulfide bonds meant that estimates of molecular weights would be considerably less than the actual molecular weights of the polypeptides in dough, it was nevertheless possible to identify specific differences in molecular size caused by TGA unambiguously.

2.3.2 HPLC

With HPLC, major advances have been made in separating wheat proteins on the basis of size (size-exclusion or SE-HPLC), charge (hydrophobic interaction chromatography or HIC, (Popineau, 1994)) and surface hydrophobicity (reversed-phase or RP-HPLC). Reports of the analysis of wheat proteins by SE- and RP-HPLC include those of Bietz (1985), Singh et al (1990), Bangur et al (1997), Cornec et al (1994), Sutton et al (1989; 1991) and Nicolas et al (1998). Although several problems with HPLC techniques have been identified and addressed (Huebner and Bietz, 1999) these methods remain invaluable in wheat protein analysis.

In this investigation, size-exclusion HPLC (SE-HPLC) and reversed-phase HPLC (RP-HPLC) were chosen for their speed and accuracy in separating wheat proteins according to size and/or charge, thus providing a useful means of detecting changes caused to the proteins of bread and croissant doughs by the addition of TGA. Accurate estimates of the molecular weights of the different fractions could be made, enabling identification of TGA- induced changes at the molecular level. Furthermore, information of a quantitative nature can be obtained by SE-HPLC, which was considered to be a useful comparison to the mainly semi-quantitative changes demonstrated by SDS-PAGE.

SE-HPLC

SE-HPLC, also called gel permeation or gel filtration, separates proteins by molecular size on a gel column (Autran, 1994). The principle of the separation is the restricted diffusion of the molecules into the gel granules depending on their porosity. Large proteins elute rapidly from the column, whereas smaller proteins are retarded and thus elute later.

The solvent system moving through the column is known as the mobile phase, whereas the porous silica or polymer supports are the stationary phase. In SE-HPLC, only one pump is required, as there is no gradient for the solvent (isocratic elution) with flow rates around 1.0 mL/minute (Autran, 1994). A typical trace shows retention time (in minutes) plotted against the UV absorbance at 210nm, the primary absorbance of peptide bonds. Peaks corresponding to individual proteins or polypeptides are displayed, which can then be quantified by calculation of the areas under the peaks. It has been reported that the presence of SDS in the solvent system is detrimental to the silica-based matrices (Pasaribu, 1992). This and other difficulties relating to the insolubility of wheat proteins have resulted in several variations of the established methods (Autran, 1994).

RP-HPLC

In RP-HPLC, proteins are separated by surface hydrophobicity. The columns generally contain spherical silica packings, usually porous, coated with various hydrophobic ligands (Lafiandra et al, 1994). The mobile phase is usually an aqueous acetonitrile (CH₃CN) containing trifluoroacetic acid (TFA) as an ion-pairing reagent. Gradient elution is typically used in order to separate proteins with a wide hydrophobicity range. A typical trace displays shows retention time (in minutes) plotted against the UV absorbance at a wavelength that can be used to quantitate proteins, with peaks representing the eluted proteins.

It was anticipated that the choice of these three methods would allow comprehensive analysis of the TGA-induced changes to the proteins of wheat-based dough.

2.4 SDS-PAGE analysis of changes to dough proteins by TGA

2.4.1 Background

While there have been many reports of the use of TGA in food products (Nonaka et al, 1992; Motoki and Seguro, 1998; Kuraishi et al, 2001), relatively few relate to those derived from wheat flour. Of these, the most prominent studies are those of Larre et al (1998; 2000 a, b) who have investigated the effect of treating extracted and freeze-dried gluten, with TGA from a *Streptoverticillium* sp. They found, in the first instance, that TGA treatment reduced the extractability of total proteins due to the formation of large insoluble polymers (Larre et al, 1998). They also showed that gluten films modified by TGA-catalysed crosslinking had improved mechanical strength (Larre et al 2000 b). Once again, large crosslinked polymers were demonstrated in the modified gluten, on SDS-PAGE, most of which could not penetrate the stacking gel. In addition to these studies, they also conducted immunoblotting studies in which antibodies directed against specific gluten peptides were used to recognise TGA reaction products. As with previous work, the proteins of gluten (gliadins and glutenins) were found to be effective substrates for TGA.

The crosslinking of gluten proteins as described above, has mainly been conducted in *in vitro* buffer systems. Therefore our work sought to investigate the crosslinking of the individual protein groups, both gluten-forming and non gluten-forming, while within the dough system, particularly in relation to the observed changes to the macroscopic properties of these foods described in section 2.1. The following sections will describe the effect of TGA on proteins of bread, croissant and biscuit dough *in situ*, as analysed by SDS-PAGE and HPLC.

2.4.2 SDS-PAGE analysis of dough proteins *in situ*

Bread, croissant and biscuit doughs were prepared with and without TGA and protein extracts from these were run on SDS-PAGE. To ensure reproducibility, at least 30-40 extractions and analyses were carried out for each dough type. Thus, conclusions were drawn from many repeated experiments and the results shown are representative examples. For each analysis, the volume of sample was adjusted according to the

protein concentration, as judged by Bradford analysis, in order to allow clear band visualisation and meaningful comparison of the size distribution of the wheat proteins.

In addition to observing specific changes to the proteins, it was of interest to study the action of TGA within the dough, over time. During proving the dough is allowed to relax following the mechanical processes such as mixing and sheeting (Siffring and Bruinsma, 1993). Some change was anticipated upon proving of the doughs, since the proving temperature of 23°C is appropriate for enzyme activity. Thus, the bread, croissant and biscuit doughs prepared for analysis were also proved for 30 minutes, following which samples were taken and analysed as before.

2.4.3 Bread and croissant dough proteins

In all of the SDS-PAGE analyses, the TGA response was found to be immediate, apparently occurring while the doughs were still in the mixer. This interesting characteristic was evident in the samples frozen immediately in liquid nitrogen upon removal from the mixer and maintained in those proved up to 30 minutes, as shown in the figures below.

Results of SDS-PAGE analyses were consistent in bread and croissants doughs, suggesting that the enzyme is not influenced by differences in the formulation of the dough, such as the presence of fat. Sizes of the reduced proteins were in general agreement with other reports of reduced wheat protein extracts. We found that reduced protein subunits isolated from the SDS-insoluble glutenins occurred within a molecular weight range from approximately 200 kDa down to 16 kDa, which compared well with the reported range of 250 to 4 kDa (Kasarda et al, 1998). Any proteins smaller than 10 kDa were eliminated from the end of the gel. SDS-soluble glutenins occurred at sizes up to 66 kDa, while gliadins featured up to 84 kDa, in agreement with Kasarda et al (1998). We found the albumin/globulin group to appear anywhere between 4 and 84 kDa although more of the bands were accumulated at the lower end of this size range, as was expected for the comparatively small size of this group (Wrigley and Bietz, 1988). The albumin bands at 84 kDa were most likely aggregates of albumin proteins that could not be broken up by SDS.

Figures 2.1- 2.4 show the albumin/globulin proteins in lanes 2 and 3 with and without TGA. On comparison, the three bands in lane 3, shown at position A, are clearly absent in lane 2. This observation, along with the appearance in lane 2 of a high molecular weight band at B with the accompanying smeared effect, indicate the formation of crosslinked protein of varying sizes (Fayle et al, 2001). Bands below 29 kDa in this fraction were found to be variable with respect to the crosslinking effect, appearing unaffected in some gels but absent in others. This may indicate differences in the availability of lysine in these proteins. In contrast to these, two intense bands just below the 36 kDa position (shown at G) were consistently unaffected by the addition of TGA to the dough.

Lanes 4 and 5 correspond to the gliadin group of dough proteins, with and without TGA. The main finding for this group was the apparent lack of crosslinking activity by TGA, as denoted by the unchanged band profiles of the TGA vs control samples. This observation was the consistent for gliadins in all the doughs analysed. However, this finding for the gliadins was contrary to the reports of Larre et al (2000 a) for their studies *in vitro*. Further investigation of this was conducted and is described in section 2.6.

SDS-soluble glutenins are shown in lanes 6 and 7. The fading or disappearance of bands in areas C and D indicates their removal through crosslinking by TGA. Crosslinked material was evident at the top of the gels in some analyses, while in others this was not seen, as in the pictures shown. This possibly indicates the formation of complexes too large for the gel pores. The two 36 kDa bands which were unaffected in the albumin fraction, were also unaffected in this group.

Figure 2.1: SDS-PAGE of bread dough extracts, frozen in liquid nitrogen immediately upon preparation. **Lane 1:** Sigma molecular weight marker; **Lane 2:** albumins/globulins from TGA dough; **Lane 3:** albumins/globulins from control dough; **Lane 4:** gliadins from TGA dough; **Lane 5:** gliadins from control dough; **Lane 6:** SDS-soluble glutenins from TGA dough; **Lane 7:** SDS-soluble glutenins from control dough; **Lane 8:** SDS-insoluble glutenins from TGA dough; **Lane 9:** SDS-insoluble glutenins from control dough.

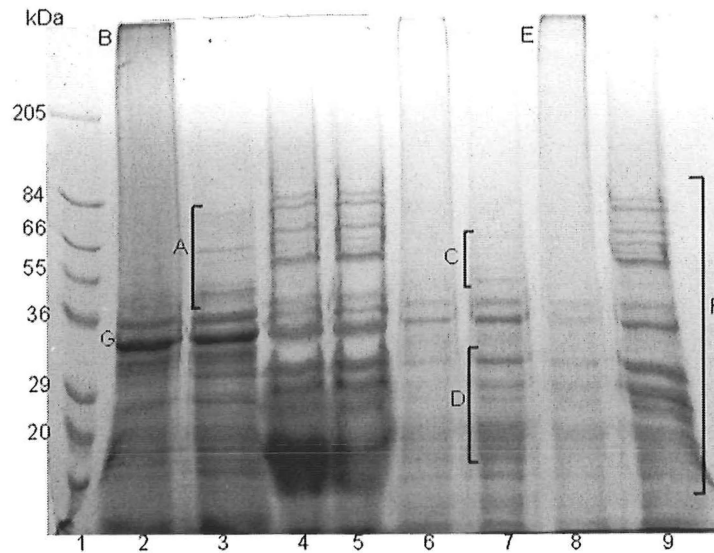


Figure 2.2: SDS-PAGE of bread dough extracts, proofed 30 minutes. **Lane 1:** Sigma molecular weight marker; **Lane 2:** albumins/globulins from TGA dough; **Lane 3:** albumins/globulins from control dough; **Lane 4:** gliadins from TGA dough; **Lane 5:** gliadins from control dough; **Lane 6:** SDS-soluble glutenins from TGA dough; **Lane 7:** SDS-soluble glutenins from control dough; **Lane 8:** SDS-insoluble glutenins from TGA dough; **Lane 9:** SDS-insoluble glutenins from control dough.

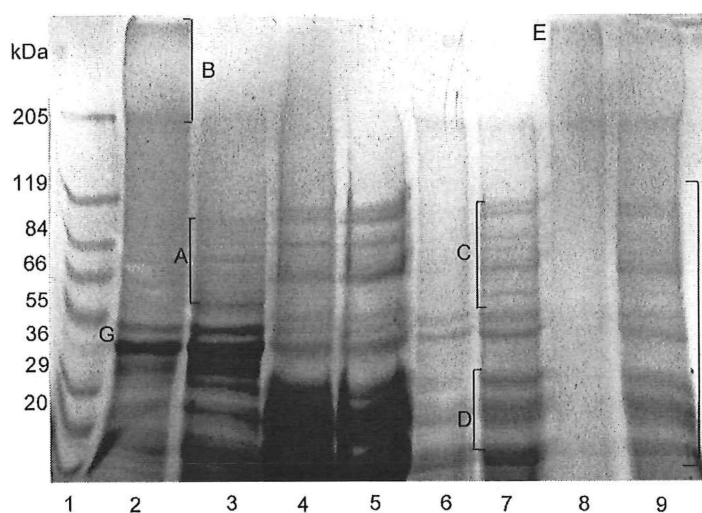


Figure 2.3: SDS-PAGE of croissant dough extracts, frozen in liquid nitrogen immediately upon preparation. **Lane 1:** Sigma molecular weight marker; **Lane 2:** albumins/globulins from TGA dough; **Lane 3:** albumins/globulins from control dough; **Lane 4:** gliadins from TGA dough; **Lane 5:** gliadins from control dough; **Lane 6:** SDS-soluble glutenins from TGA dough; **Lane 7:** SDS-soluble glutenins from control dough; **Lane 8:** SDS-insoluble glutenins from TGA dough; **Lane 9:** SDS-insoluble glutenins from control dough.

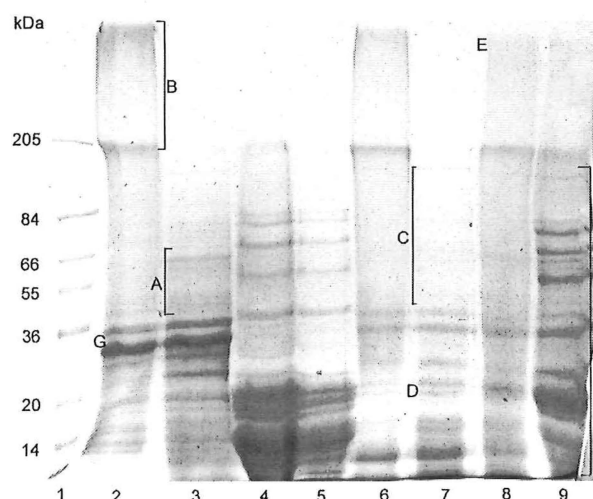
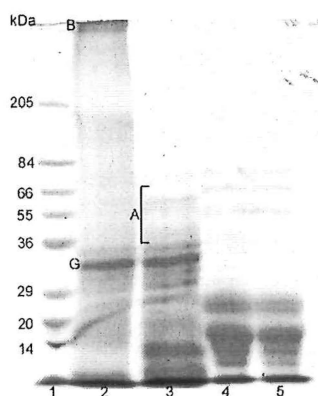
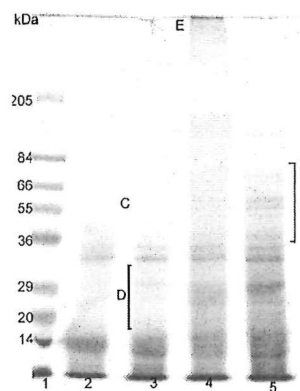


Figure 2.4 (a): SDS-PAGE of albumin/globulin and gliadin croissant dough extracts, proofed 30 minutes. **Lane 1:** Sigma molecular weight marker; **Lane 2:** albumins/globulins from TGA dough; **Lane 3:** albumins/globulins from control dough; **Lane 4:** gliadins from TGA dough; **Lane 5:** gliadins from control dough. **(b)** SDS-soluble and insoluble glutenin croissant extracts proofed 30 minutes. **Lane 1:** Sigmamarker; **Lane 2:** SDS-soluble glutenins from TGA dough; **Lane 3:** SDS-soluble glutenins from control dough; **Lane 4:** SDS-insoluble glutenins from TGA dough; **Lane 5:** SDS-insoluble glutenins from control dough.

(a)



(b)



The most dramatic effect of TGA was observed on the polymeric proteins dispersed by sonication, namely the high molecular weight glutenins. The consistently exhibited effect was the almost complete disappearance of all the bands in the TGA lane of this fraction (lane 8) as compared to the control (labelled F, lane 9). Of all the changes to the dough protein fractions, this was the most obvious indicator of the action of TGA at the molecular level. An added feature of the TGA lane of this fraction was the presence of amorphous material giving the lane a smeared appearance. This also implied the aggregation of polymers as a result of crosslinking and the possibility of material too large to enter the gel as seen in the stacking gel in some results. Associated with these striking effects, new bands appeared, as seen at position E.

Thus, it seems that the SDS-insoluble glutenins and the albumins and globulins are crosslinked by TGA. In terms of improvements to wheat products, it is the observed response of the large, insoluble glutenins that is most likely to be significant. Since the high molecular weight glutenins have been previously shown to be most indicative of bread quality, the crosslinking of these large proteins by TGA into even larger complexes could account for the greatly increased dough strength of TGA-treated bread and the dramatic increase to the volume of layered pastries such as croissants. The crosslinking of the non-dough forming albumin/globulin group on the other hand, may be responsible for the improvements in crumb strength and texture.

This is corroborated by recent research in our laboratory by Gerrard et al (2002 a, b, c), in which it was demonstrated that crosslinking of wheat proteins by Maillard-type chemistry, using several carbonyl compounds, including glutaraldehyde, could occur within a dough system. Studies were conducted *in situ* with doughs that had been prepared with several concentrations of the crosslinking agent, and the resulting macroscopic observations were related to studies at the molecular level. Bread loaves prepared with additives such as formaldehyde and glutaraldehyde were found to have increased relaxation times compared to controls containing no additive. This indicated increased dough development, presumed to be due to the strengthening of the gluten network through the addition of Maillard-type crosslinks. However, the addition of glutaraldehyde produced loaves that decreased in volume. These loaves were otherwise of acceptable quality, with good crumb strength and texture. Croissant volume was also unaffected by the addition of glutaraldehyde. Investigations of these results at the

molecular level revealed that, at a concentration of 200 p.p.m, glutaraldehyde crosslinked only the albumin and globulin fraction of dough proteins, while the gliadins and the glutenins were unaffected. It appears, then, that the crosslinking of the albumins and globulins strengthens the gluten network with the result of improving crumb strength and texture, whereas increases in product volume, particularly in pastries, will only occur as the result of the crosslinking of the SDS-insoluble glutenin fraction.

With respect to the difference in volume of TGA-treated bread vs. croissants, clearly while both types of dough exhibited the crosslinking of the SDS-insoluble glutenins by TGA, there was a difference in the macroscopic manifestation of this effect. This difference may be attributed to the layered nature of a croissant dough producing a more exaggerated effect upon gas cell expansion than the three-dimensional structure of bread dough. Laminated pastries such as croissants require rigid layers between the gas-filled fat layers, so strengthening of the dough layers with TGA is a desirable effect. On the other hand, the requirement in bread is for a flexible dough that would allow gas cell expansion, thus increased dough strength is not necessarily beneficial.

These results led to the conclusion that the action of TGA is specific to certain wheat protein substrates in dough. In spite of variations due to solubility problems, particularly in the SDS-soluble and insoluble glutenins, the same proteins were found to be crosslinked in bread and croissants, while those that were not affected remained as such.

It is essential that a potential additive to a dough system be considered within the context of the procedures to which it is subjected. Doughs of bread and other baked products are commonly exposed to varying conditions prior to the baking process. These may be intrinsic, including variations in the quality and proportions of the ingredients and additives, or extrinsic, pertaining to differences in handling, proofing and long-term storage. Proofing, or resting of doughs at a constant temperature before baking is an essential step in the fermentation stage of breadmaking, ensuring an even distribution of the gas cells that develop as a result of yeast activity throughout the product. The lack of further reaction on proofing, illustrates the stable, irreversible nature of the reaction, which is an advantage to the baking industry.

Also with regard to variables, intrinsic variations in the ingredients often occur. As well as these, doughs of breads and croissants are being increasingly frozen to meet consumer demands and the export market. Therefore, in addition to the effect of proofing, we also investigated enzyme dose and long-term frozen storage, for their effect on the crosslinking reaction. This is addressed in *Chapter three*.

2.4.4 Biscuit dough proteins

Gels of biscuit protein extracts showed some differences to those of the bread and croissants. In particular, the protein profile of the albumin/globulin group in some gels showed some extra bands (figure 2.5 bands I, J, H and O), which were most likely to be the egg proteins in the biscuit formulation. Particularly, a band likely to be ovalbumin (figure 2.5, band labelled O) occurred at approximately 45 kDa. Separate runs of ovalbumin alone on SDS-PAGE confirmed the 45 kDa position of this protein. This protein was only slightly affected by TGA, as evidenced by the lessening of band intensity of the gel. This was consistent with ovalbumin being a globular protein known to be a poor substrate for TGA (Ikura et al, 1984).

Since TGA does not improve the quality of biscuits (Gerrard et al, 1998 b) and had no novel molecular effects, no further experiments were carried out on biscuit dough.

2.4.5 Effect of enzyme concentration on the crosslinking reaction

The enzyme dosage for bread, croissant and biscuits was set at 5000 p.p.m. This concentration was based on earlier studies (Gerrard et al, 1996) and was decided upon based on the fact that the commercial preparation of TGA consisted of only 20% enzyme, and lower doses did not produce desirable effects in bread and croissants.

Figures 2.6 and 2.7 show the effect in each protein group, of doubling of the enzyme dose. It is evident that, the response at the lower concentration is sufficient to bring about the full effect and that increasing the enzyme does not appear to add to it. Thus, as with proofing time, the initial reaction is maintained even with a higher enzyme concentration.

Figure 2.5: SDS-PAGE of biscuit dough extracts, frozen in liquid nitrogen immediately upon preparation. **Lane 1:** Sigma molecular weight marker; **Lane 2:** albumins/globulins from TGA dough; **Lane 3:** albumins/globulins from control dough; **Lane 4:** gliadins from TGA dough; **Lane 5:** gliadins from control dough; **Lane 6:** SDS-soluble glutenins from TGA dough; **Lane 7:** SDS-soluble glutenins from control dough; **Lane 8:** SDS-insoluble glutenins from TGA dough; **Lane 9:** SDS-insoluble glutenins from control dough.

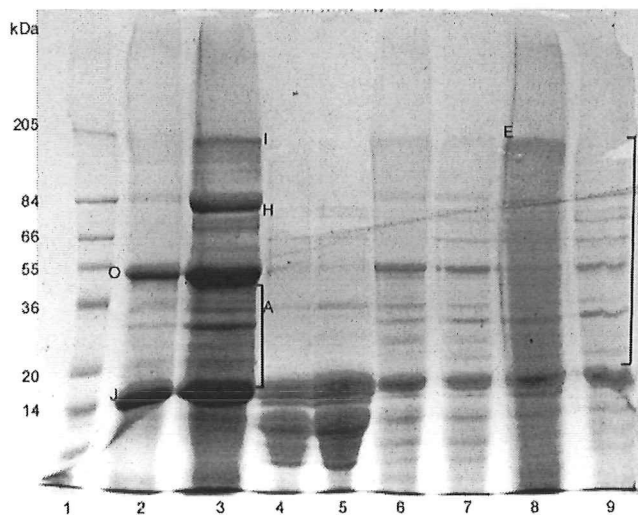


Figure 2.6: SDS-PAGE of bread albumin and gliadin extracts from control doughs and those prepared with 1x (5000 p.p.m) and 2x (10,000 p.p.m) TGA. **Lane 1:** Sigma marker; **Lane 2:** control albumins/globulins; **Lane 3:** albumins/globulins with 1x TGA; **Lane 4:** albumins/globulins with 2x TGA; **Lane 5:** Sigmamarker; **Lane 6:** control gliadins; **Lane 7:** gliadins with 1x TGA; **Lane 8:** gliadins with 2x TGA.

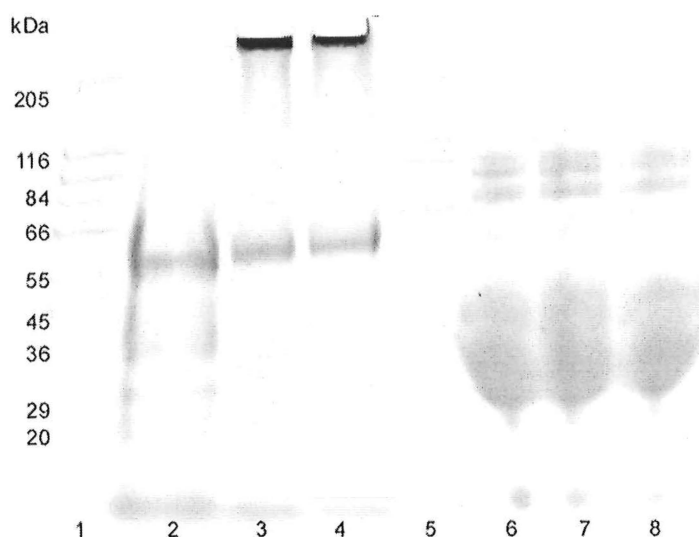
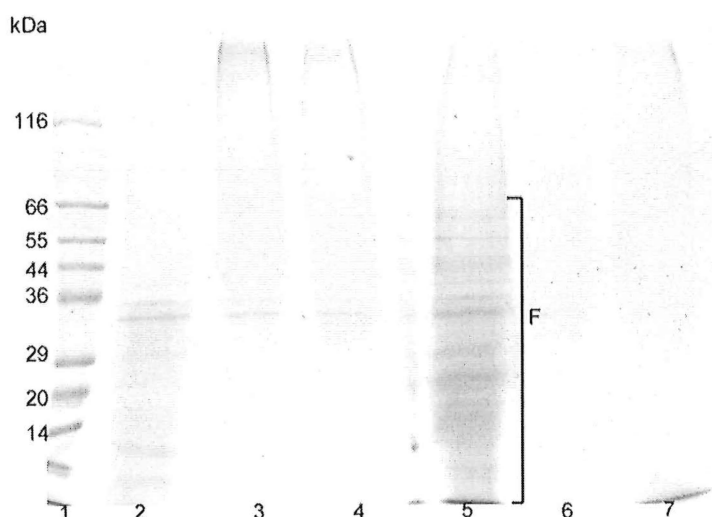


Figure 2.7: SDS-PAGE of bread SDS-soluble and insoluble glutenin extracts from control doughs and those prepared with 1x (5000 p.p.m) and 2x (10,000 p.p.m) TGA. **Lane 1:** Sigma marker; **Lane 2:** control SDS-soluble glutenins; **Lane 3:** SDS-soluble glutenins with 1x TGA; **Lane 4:** SDS-soluble glutenins with 2x TGA; **Lane 5:** control SDS-insoluble glutenins; **Lane 6:** SDS-insoluble glutenins with 1x TGA; **Lane 7:** SDS-insoluble glutenins with 2x TGA.



2.4.6 Substrate specificity of TGA

In order to explain the specificity of the TGA-catalysed crosslinking reaction for certain classes of wheat protein, the crosslinking reaction was considered. The acyl transfer reaction catalysed by TGA occurs between the γ -carboxyamide group of peptide-bound glutamine residues, and primary amines, particularly lysine (refer figure 1.9, *Chapter One*). While gliadins and glutenins are high in glutamine, they are known to be limited in their lysine content (Wrigley & Bietz, 1988). By comparison, albumins have a higher lysine content than gliadins (refer table 2.1) and are therefore more likely to undergo TGA- mediated crosslinking. It was therefore, somewhat surprising that the insoluble glutenin group, with a limited lysine content, displayed such a pronounced effect with TGA, *in situ*. This is explored further in *Chapter Five*.

Table 2.1: Lysine content (mol %) of wheat protein fractions (Eynard et al, 1994)

Wheat protein	Lysine (mol %)
Albumins / globulins	3.2
Gliadins	0.3 - 0.7
Glutenins (LMW)	0.6-1.0
Glutenins (HMW)	1.1-1.8

The observed response of the high molecular weight glutenins was compatible with the *in vitro* studies of Larre et al (2000 a, b), who noted that whilst all the constituent gluten proteins acted as suitable substrates for TGA, the HMW glutenins demonstrated the highest activity, as judged by the selective recognition by antibodies of mainly HMW glutenin peptides in the polymeric reaction products. However, our findings for the gliadin group were contrary to the findings of Larre et al (2000 a). In our studies, this group consistently showed no response to TGA treatment of the doughs. Prior to considering this further, confirmation for the above results was sought by HPLC.

2.5 HPLC analysis of changes to dough proteins by TGA

2.5.1 RP-HPLC analysis of dough proteins *in situ*

RP-HPLC was carried out on the albumin/globulin, gliadin and SDS-soluble and insoluble glutenin groups for control and TGA-treated doughs. Clear differences were evident between control and TGA samples.

RP-HPLC results showed major reductions in the sizes of several peaks, in all but the gliadin fraction. Figure 2.8 of the albumins and globulins shows the reduction of peaks particularly in the region between 15 and 30 minutes upon TGA treatment. No major changes are evident in figure 2.9 of the gliadins, between control and TGA containing dough samples. Figure 2.10 shows reduction in peak sizes in SDS-soluble glutenins, particularly at 19 minute retention time. The largest effect of TGA is seen in figure 2.11, of the SDS-insoluble glutenins. In this trace, major reductions of the peaks are evident throughout the whole elution period. RP-HPLC traces of croissant protein extracts are shown in the Appendix.

These results were entirely consistent with the SDS-PAGE results. Unfortunately, reproducibility of RP-HPLC proved difficult, therefore our attention turned to SE-HPLC for further analysis.

Figure 2.8: RP-HPLC traces of the albumin / globulin fraction from TGA-treated (upper) and control (lower) bread doughs, proofed 30 minutes.

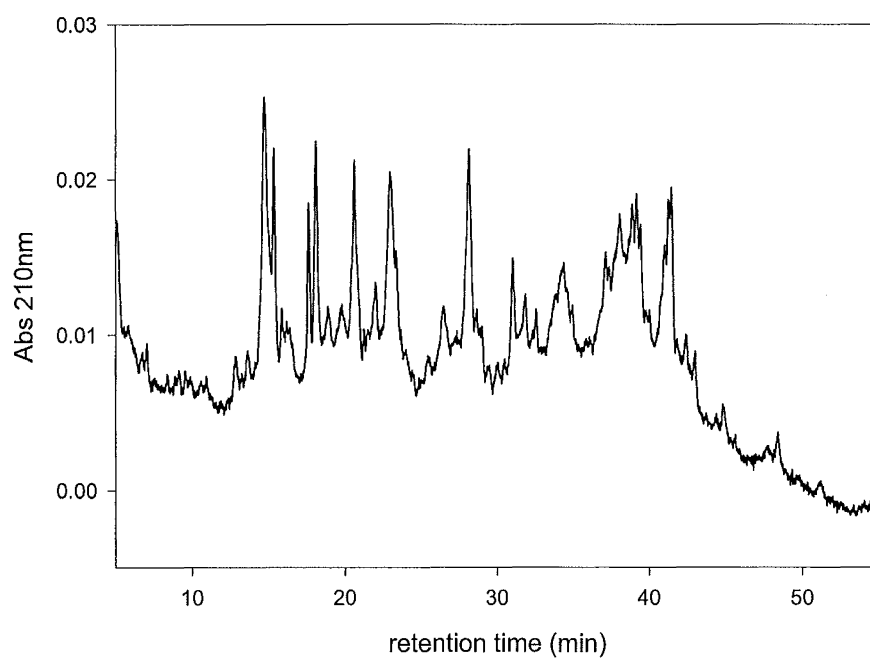
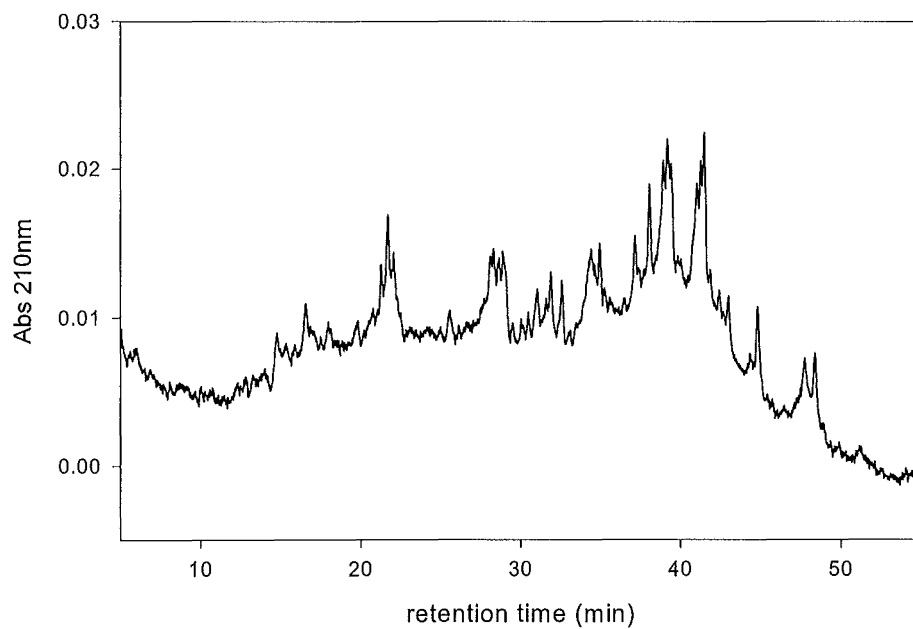


Figure 2.9: RP-HPLC traces of the gliadin fraction from TGA-treated (upper) and control (lower) bread doughs, proofed 30 minutes.

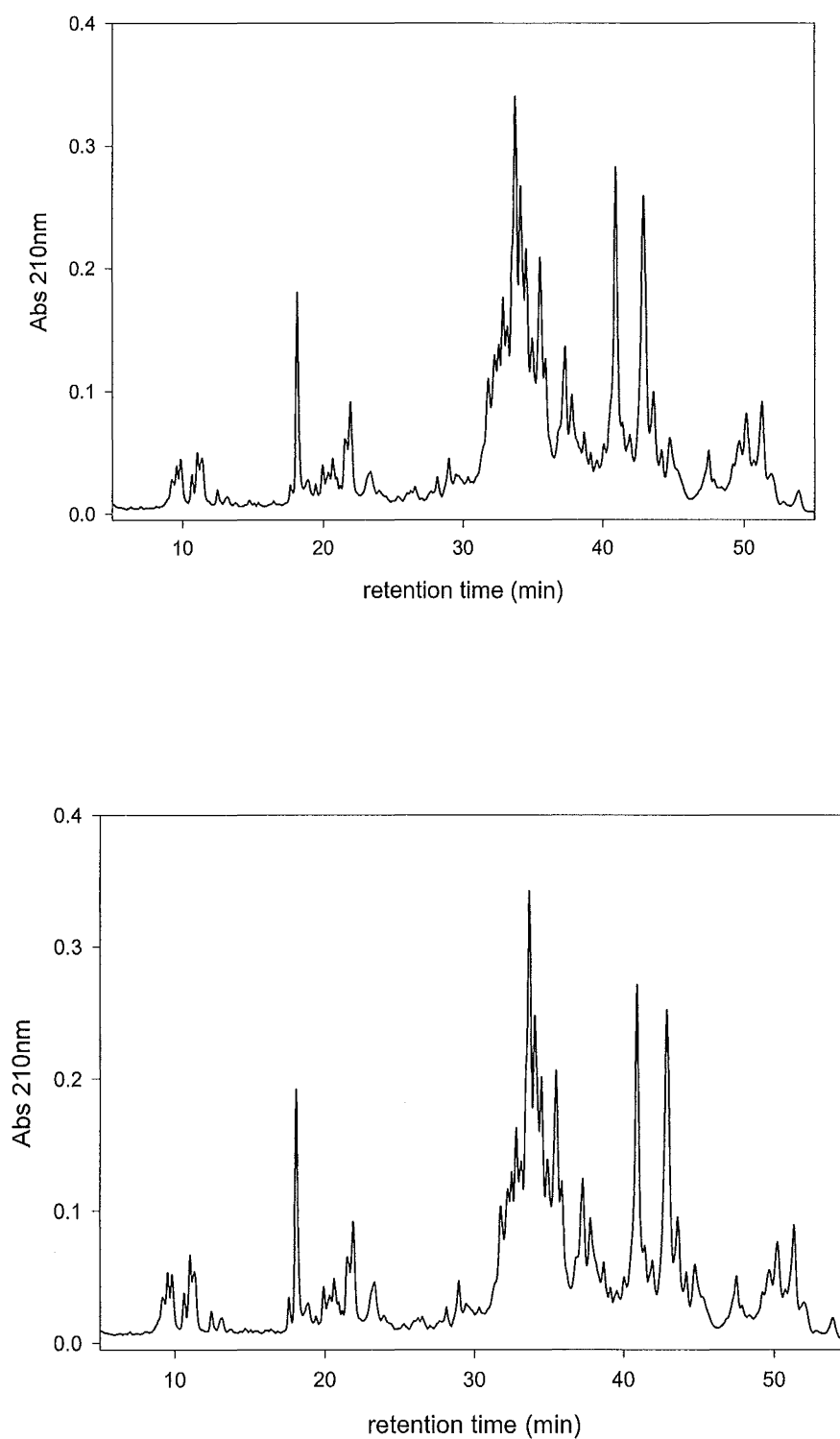


Figure 2.10: RP-HPLC traces of the SDS-soluble glutenin fraction from TGA-treated (upper) and control (lower) bread doughs, proofed 30 minutes.

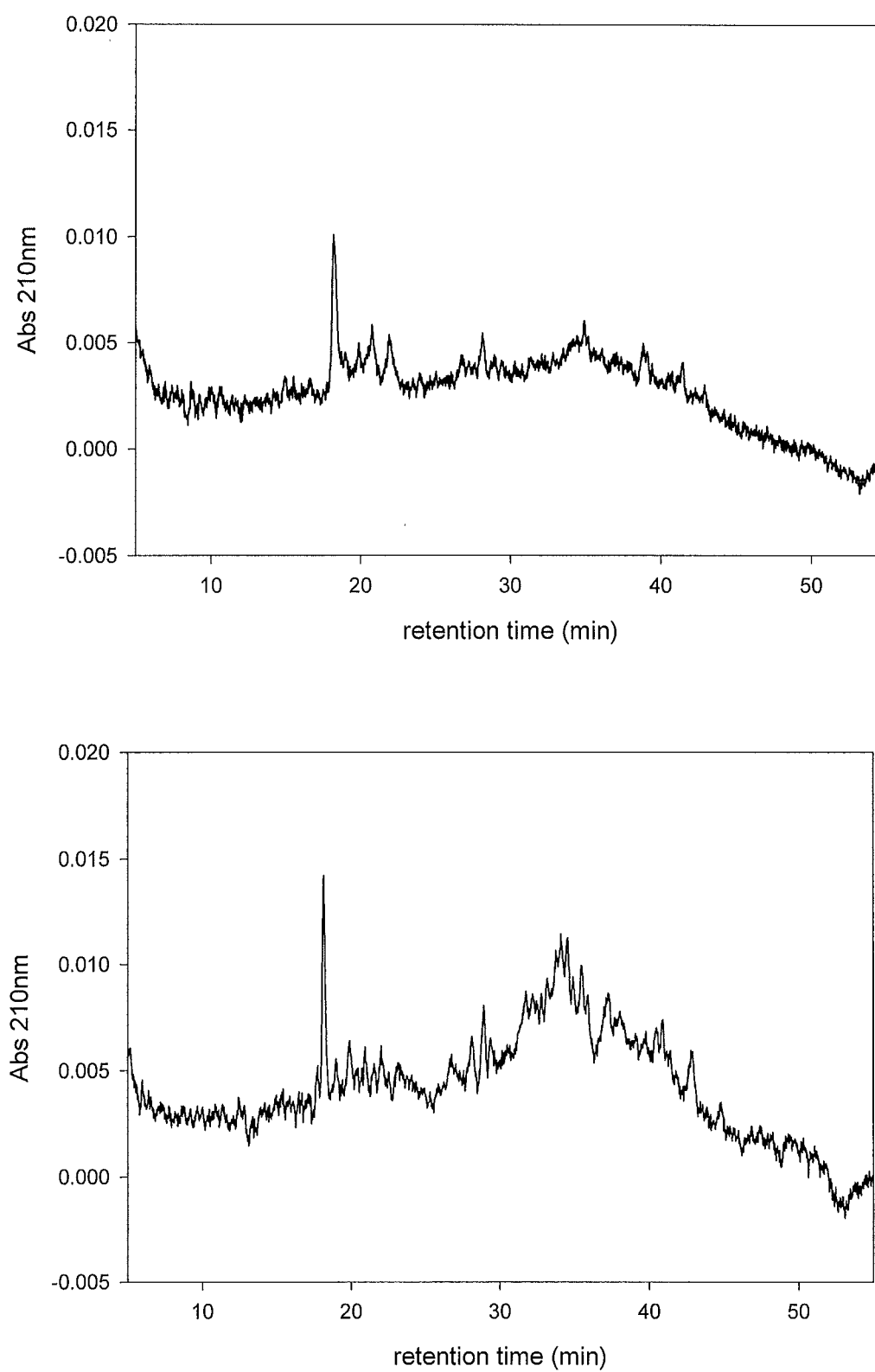


Figure 2.11: RP-HPLC traces of the SDS-insoluble glutenin fraction from TGA-treated (upper) and control (lower) bread doughs, proofed 30 minutes.

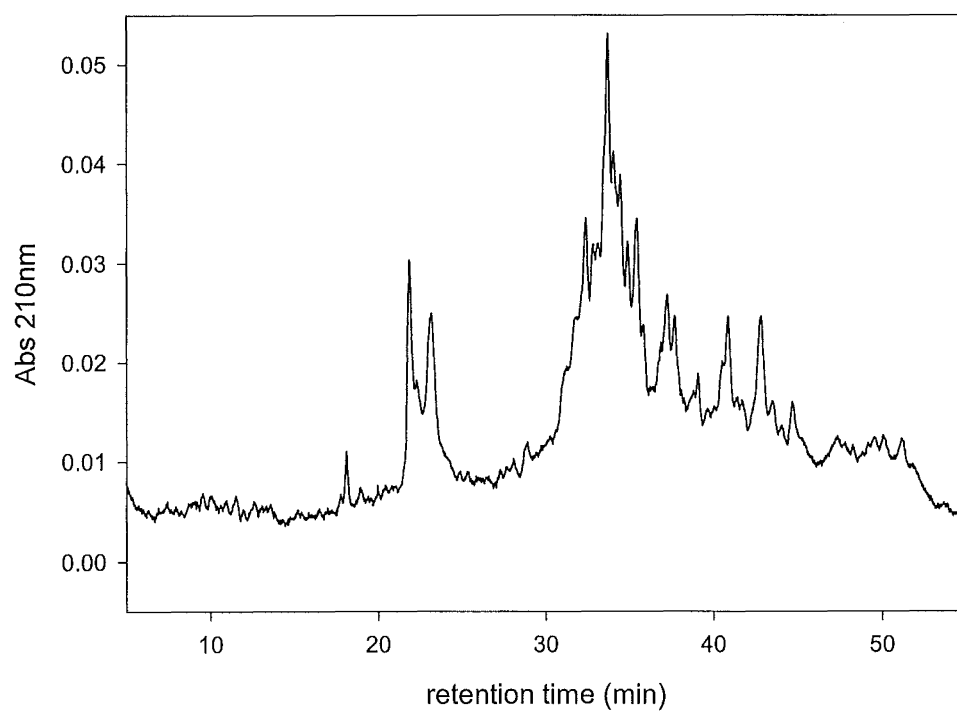
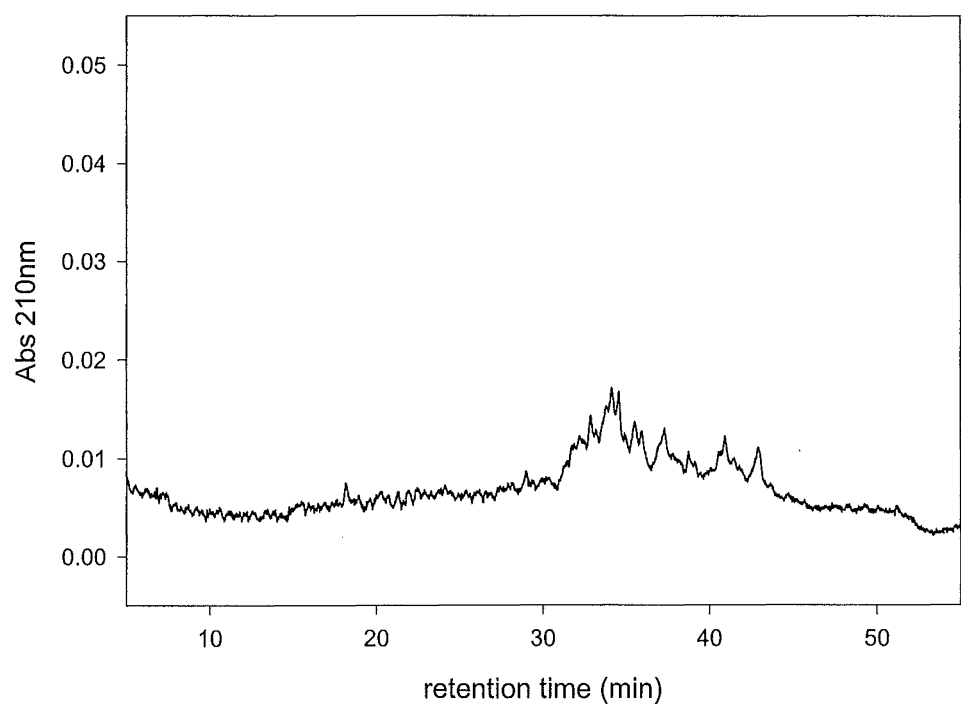


Figure 2.12: SE-HPLC traces of the albumins and globulins from TGA-treated (upper) and control (lower) bread doughs, proofed 30 minutes.

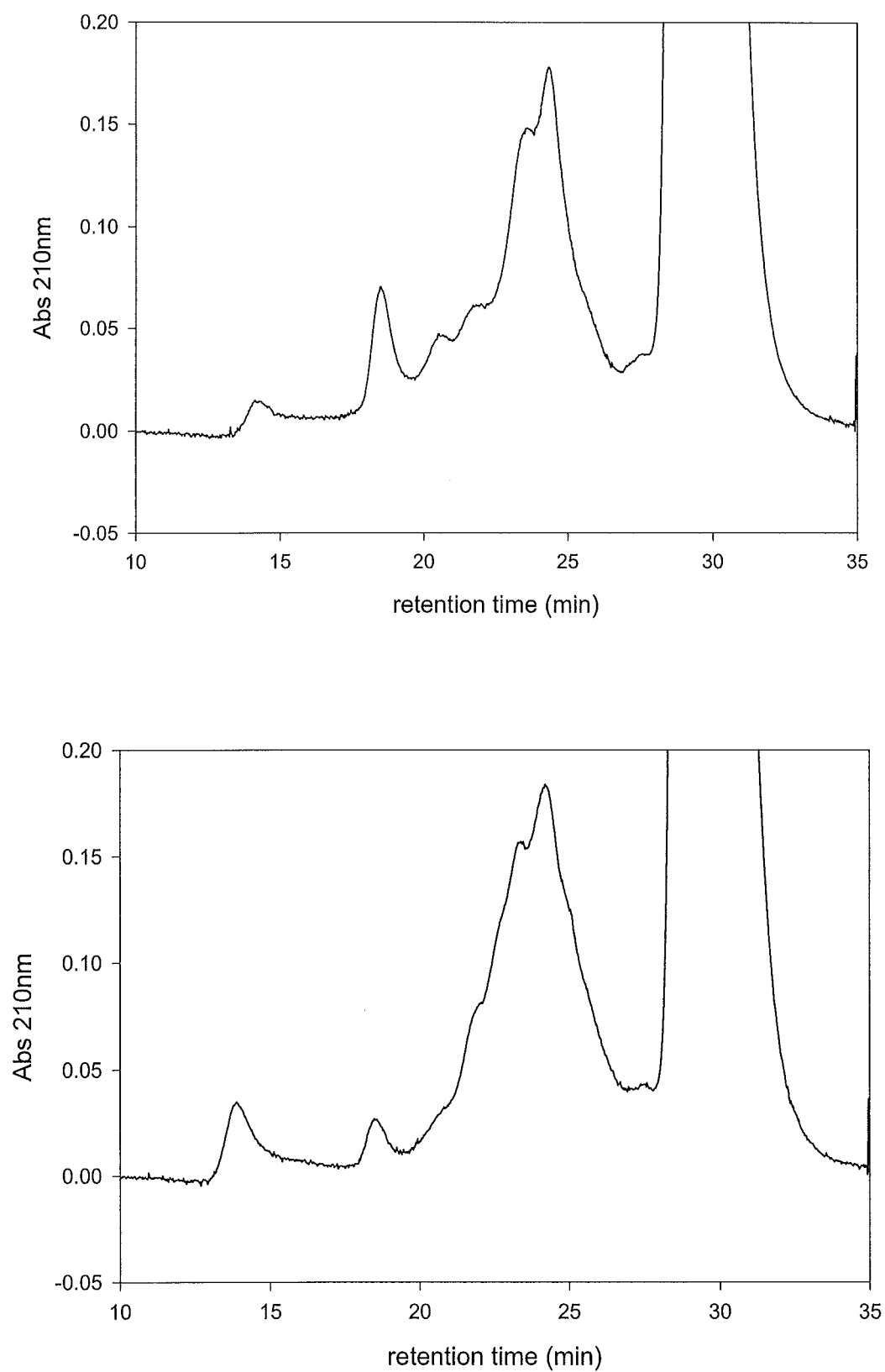


Figure 2.13: SE-HPLC traces of the gliadins from TGA-treated (upper) and control (lower) bread doughs, prooved 30 minutes.

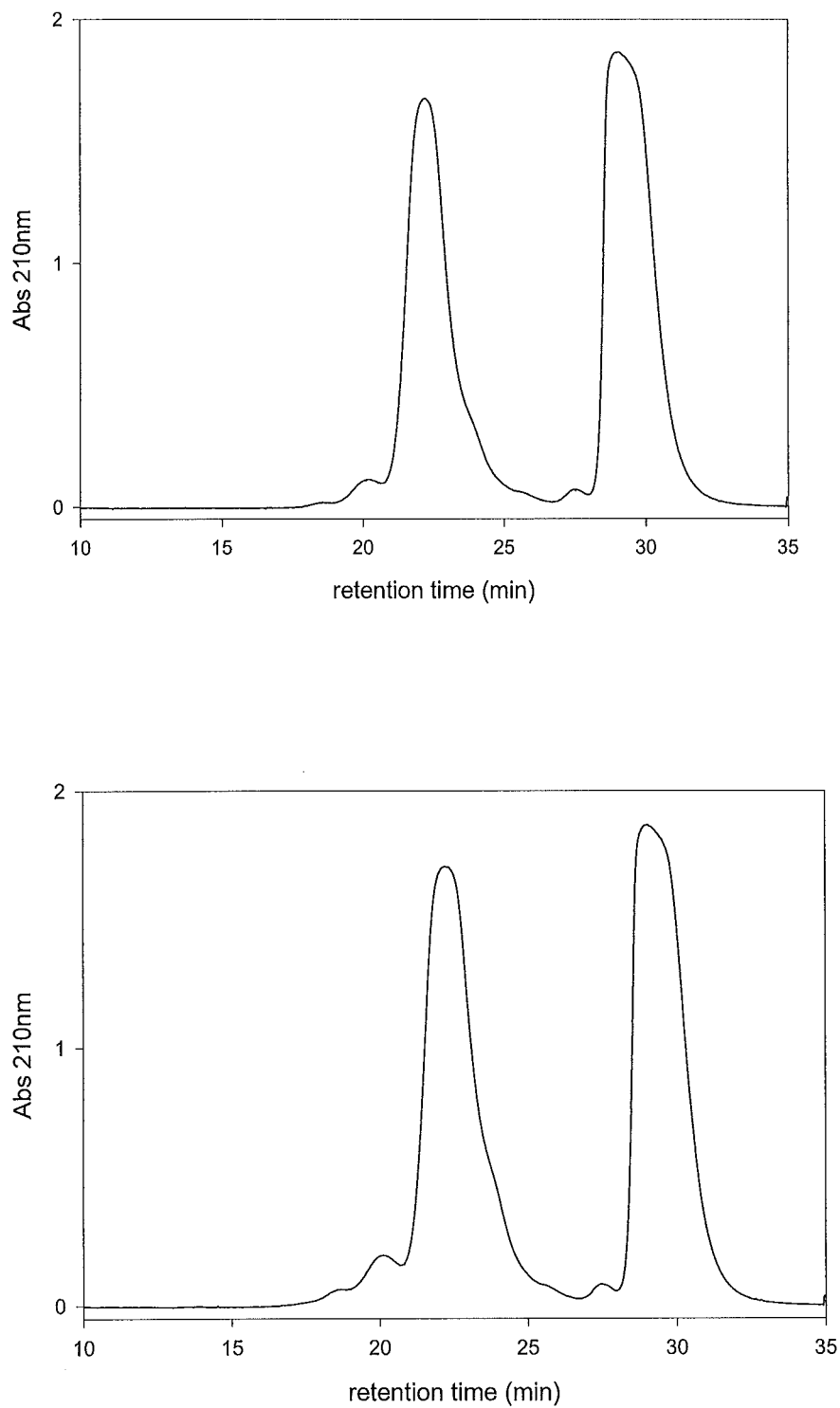


Figure 2.14: SE-HPLC traces of the SDS-soluble glutenins from TGA-treated (upper) and control (lower) bread doughs, proofed 30 minutes.

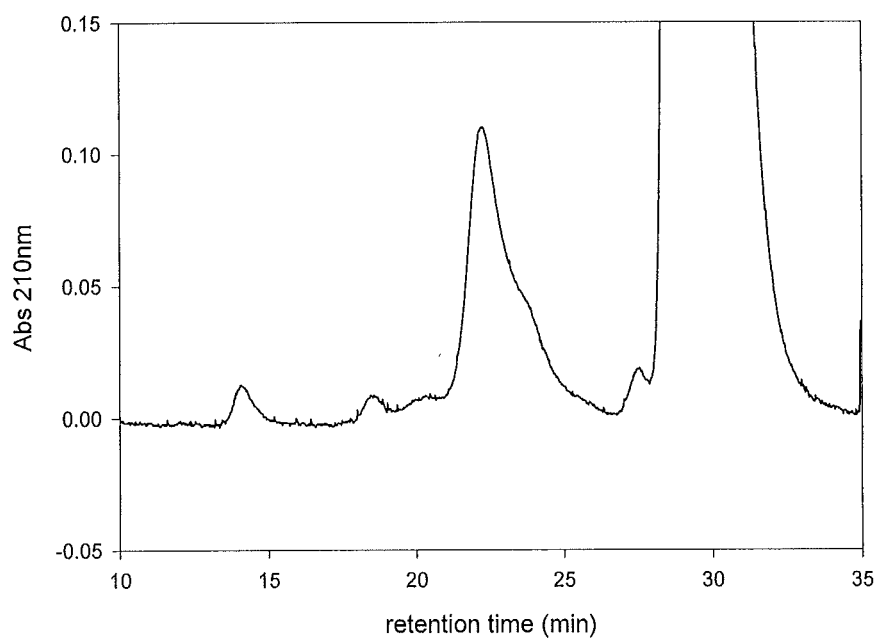
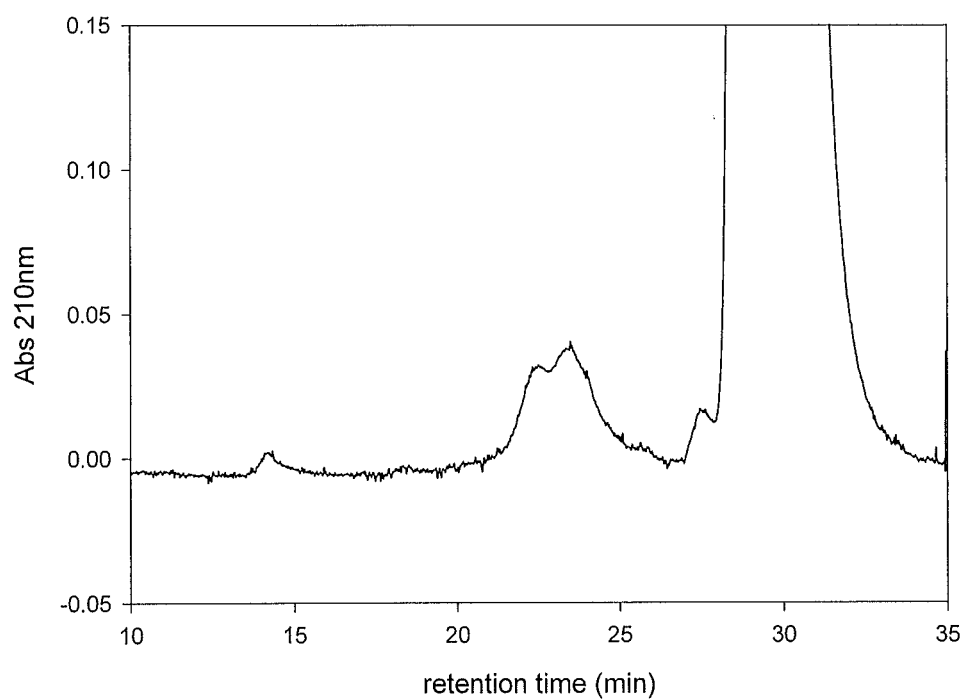
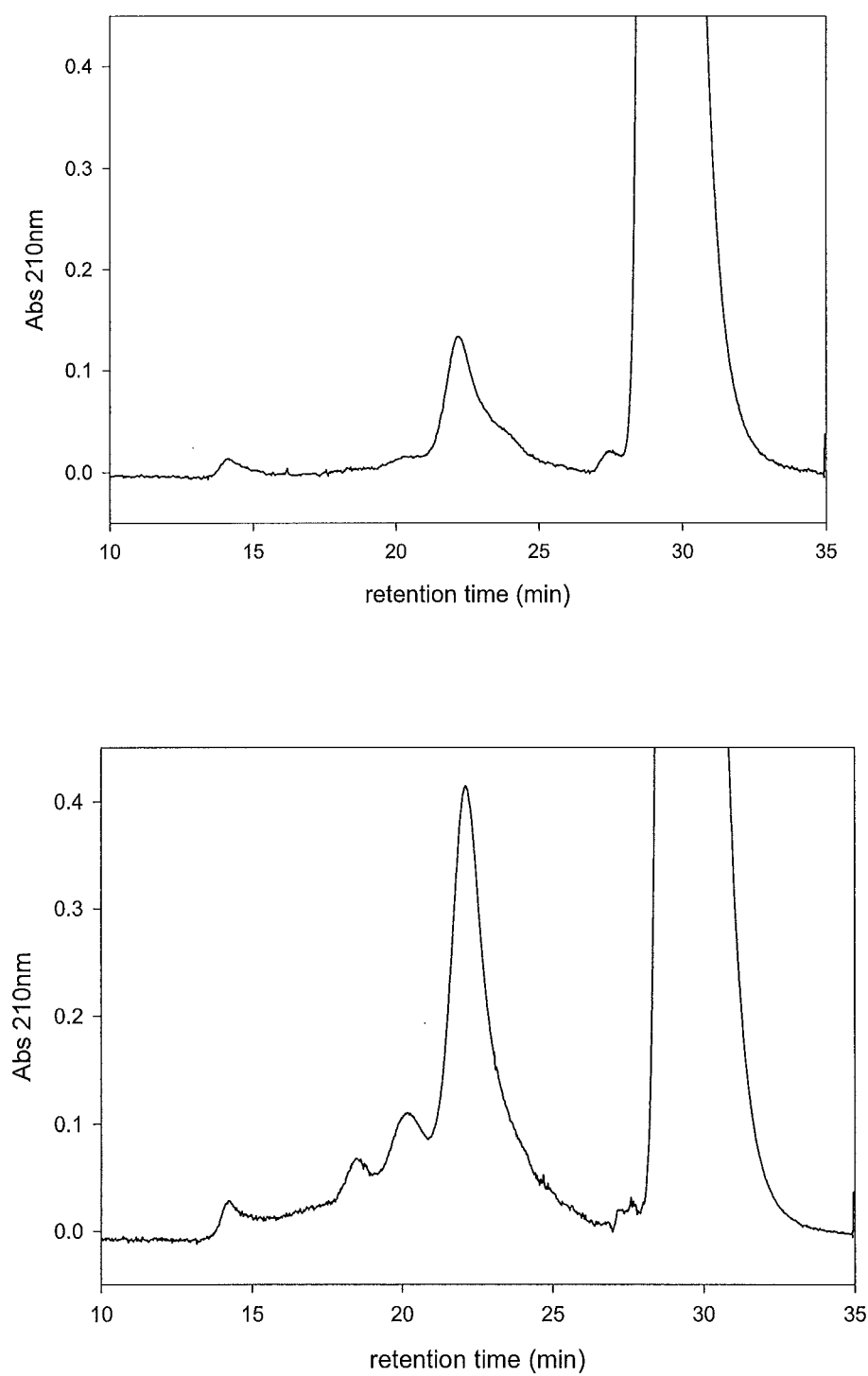


Figure 2.15: SE-HPLC traces of the SDS-insoluble glutenins from TGA-treated (upper) and control (lower) bread doughs, proofed 30 minutes.



2.5.2 SE-HPLC analysis of dough proteins *in situ*

Figure 2.12 shows an SE-HPLC trace of the albumins and globulins from control and TGA-treated bread. Reduction in peak sizes can be seen mainly in peaks up to 20 minutes retention time. As with RP-HPLC and SDS-PAGE analyses, figure 2.13 shows that the gliadin fraction has not been affected by the addition of TGA to the dough. In contrast, the SDS-soluble and insoluble glutenins (figures 2.14 and 2.15) demonstrate a large change in the main peaks due to crosslinking by TGA. SE-HPLC results for representative croissant samples are shown in the Appendix.

To quantify the changes seen on SE-HPLC between control and TGA samples of bread, croissants and biscuits, the areas below the peaks were calculated for each trace, to obtain a total value for that fraction. The difference between the control and TGA value was expressed as the % change in extractability. With the exception of the gliadins, a reduction in extractability was observed in the TGA samples, as shown in table 2.2.

Table 2.2: Decrease in protein extractability (%) due to treatment with TGA, from analysis by SE-HPLC. (*sem* = standard error of the mean of four measurements).

Fraction	Bread	Croissants
albumins / globulins	17.7 (<i>sem</i> 2.8)	31.2 (<i>sem</i> 3.0)
gliadins	-7.6 (<i>sem</i> 3.4)	-7.0 (<i>sem</i> 3.5)
SDS-soluble glutenins	23.8 (<i>sem</i> 8.4)	34.4 (<i>sem</i> 2.4)
SDS-insoluble glutenins	62.9 (<i>sem</i> 4.3)	44.7 (<i>sem</i> 6.8)

These results show that the percentage of protein occurring in each fraction has been changed in the TGA treated doughs. A decrease in the extractability of a particular fraction (e.g. in the SDS-insoluble glutenins) is a possible indicator of the formation of enormous complexes, too large to extract. On the other hand, an increase in extractability may mean that proteins that were originally found in the albumins/globulins, for example, are being extracted in the gliadin fraction following TGA treatment.

With reference to the gliadin fraction, it is evident that the amount of extractable protein increases, in contrast with the other groups where it decreases. This is consistent with the lack of response of the gliadins as seen on SDS-PAGE, and reflects a lack of reactivity *in situ*. These results were also consistent with the SE-HPLC results obtained with the analysis of control and TGA-treated croissants in frozen storage for up to three months, in which a lesser crosslinking effect was observed in the gliadin fractions (see *Chapter Three*).

Extractability of the glutenin proteins has also been studied by Huang and Khan (1997 a, b, c) in relation to the breadmaking quality of different flour varieties. Fractionation and solubility studies (Huang and Khan, 1997 a, b) showed that the stronger the wheat, the more proteins are left in the residue after the extraction, i.e. the less soluble are the component proteins. Multistacking SDS-PAGE and analysis by densitometry (Huang and Khan, 1997 c) also showed that glutenins from flours with better baking quality produced larger aggregates in the stacking gels. This was also consistent with the findings of Larre et al (1998) who reported the reduced extractability of proteins from glutens treated with TGA. It is however, noted that this correlation is stronger in wheats from USA and Australia, where there is a consistent subunit background. The correlation is somewhat reduced in NZ wheats (Sutton, 2002).

2.5.3 Summary of HPLC results

The HPLC results indicated that the albumin and glutenin groups were crosslinked by TGA, whereas the gliadins appeared largely unaffected. As with SDS-PAGE results, this was contrary to the reports of Larre et al (2000 a) with regard to the gliadins, but agreed with their findings for the HMW glutenins. Therefore, an investigation was undertaken to establish the response of the individual groups outside of the dough, *in vitro*.

2.6 Changes to dough proteins mediated by TGA *in vitro*

TGA-induced polymerisation has been demonstrated *in vitro* with several food protein substrates including those of milk (Matsumura et al (1996), soybean (Siepaio and Meunier, 1995) wheat (Alexandre et al, 1993; Larre et al, 2000 b) and even mixtures of different proteins (Motoki and Nio, 1983). In the study of Aboumahmoud and Savello (1990) with whey proteins, an increase in the crosslinking reaction was noted over time, while Larre et al (2000 a) reported the quantity of polymerised products of gluten proteins incubated with TGA to be related to both the time of incubation and enzyme concentration.

In our study, freeze-dried wheat protein extracts from control bread and croissant doughs were prepared and incubated with TGA in a Tris-HCl (pH 6.8) buffer. Reproducibility was ensured by several repetitions of each incubation and analysis by SDS-PAGE. Due to concerns about the possible damaging effects of freeze-drying on the proteins, the experiments were also carried out with fresh protein extracts from the control doughs, incubated with TGA in their respective extraction solvents. As identical results were obtained with both methods, only those relating to the freeze-dried proteins are presented.

2.6.1 Albumins and globulins

Figures 2.16 and 2.17 below for albumins and globulins from bread and croissants show that in the TGA lanes, some albumin/globulin bands have been removed from area X in the control lane (lane 2), while others are unaffected. Polymeric aggregates (labelled Y) were seen in all the TGA lanes. As with *in situ* analyses, the albumin/globulin fraction was found to be an effective substrate for TGA *in vitro*. Bands that were not removed by incubation with TGA were analogous to those found at around 36 kDa in the *in situ* analyses. Clearly, extraction from dough did not affect the status of these proteins as substrates for TGA.

Figure 2.16: SDS-PAGE of bread albumins/globulins incubated with TGA *in vitro*. **Lane 1:** Sigma marker; **Lane 2:** Control; **Lane 3:** incubation time 5 minutes; **Lane 4:** incubation time 15 minutes; **Lane 5:** incubation time 30 minutes; **Lane 6:** incubation time 1 hour; **Lane 7:** incubation time 24 hours.

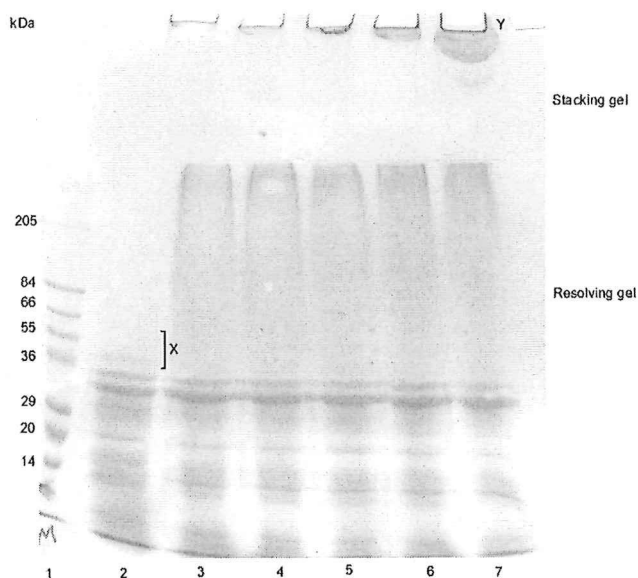
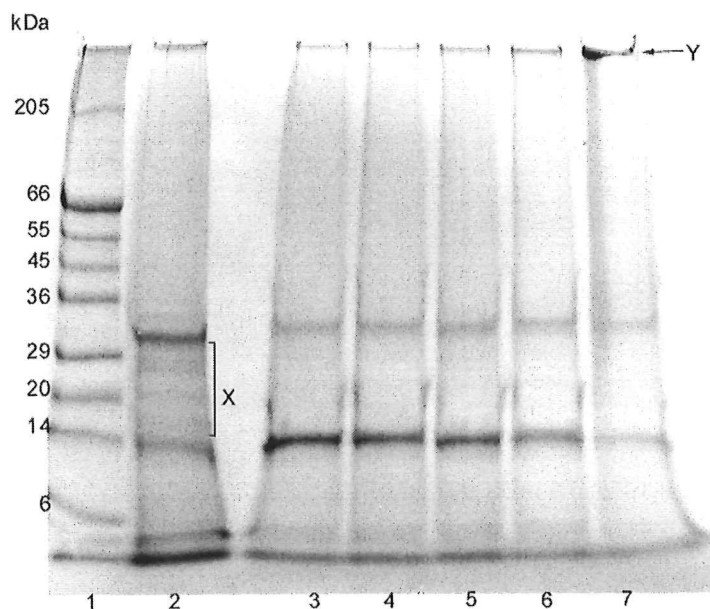


Figure 2.17: SDS-PAGE of croissant albumins/globulins incubated with TGA *in vitro*. **Lane 1:** Sigma marker; **Lane 2:** Control; **Lane 3:** incubation time 5 minutes; **Lane 4:** incubation time 15 minutes; **Lane 5:** incubation time 30 minutes; **Lane 6:** incubation time 1 hour; **Lane 7:** incubation time 24 hours.



2.6.2 Gliadins

The most interesting findings of the *in vitro* studies were those concerning the gliadins. In sharp contrast to our results *in situ* and in agreement with those of other research conducted *in vitro*, the gliadins were crosslinked very quickly by TGA, (figures 2.18 and 2.19) as evidenced by the disappearance of bands from the control lane (X) and polymeric aggregation in TGA lanes (Y) of both bread and croissant extracts.

On the basis of our findings for the gliadins within and outside the dough system, we propose that there may be factors additional to the number of reactive groups in the wheat proteins in determining the extent of crosslinking. While specific gliadins have been shown to influence breadmaking quality (van Lonkhuijsen et al, 1992), they are also most notably deficient among gluten proteins in the amino acid lysine (Wrigley and Bietz, 1988). Therefore, the occurrence of TGA-mediated crosslinking in the buffer in spite of this deficiency, suggests that the reactive groups may be less accessible to the enzyme within a dough, than *in vitro*. As well as this, the pH of a dough system being around 5.5, lysine residues may not be as available for reaction as they would in a buffer system of pH 6.8. Thus it appears that the availability of the lysine residues, due to the conformation of the gliadins as well as pH of the dough environment, is a key factor in the observed differences.

This is consistent with the reported importance of the surface properties of the gluten proteins in breadmaking. In the study of Scardone et al (2000), an immunochemical agent FF18 was employed to detect the availability of primary amino groups on the surface of gluten proteins. They demonstrated that the availability of these groups increases in conditions where gliadins and glutenins are solubilised or finely dispersed. Further support for this proposition is added by Matsumura et al (1996), who reported the milk protein α -lactalbumin to be more susceptible to TGA when in the molten globule state, caused by the disruption of intramolecular disulfide bridges and chemical modification.

Based on our findings, figure 2.20 represents the difference in the response of the gliadins to TGA within and outside of the dough system.

Figure 2.18: SDS-PAGE of bread gliadins incubated with TGA *in vitro*. **Lane 1:** Sigma marker; **Lane 2:** Control; **Lane 3:** incubation time 5 minutes; **Lane 4:** incubation time 15 minutes; **Lane 5:** incubation time 30 minutes; **Lane 6:** incubation time 1 hour; **Lane 7:** incubation time 24 hours.

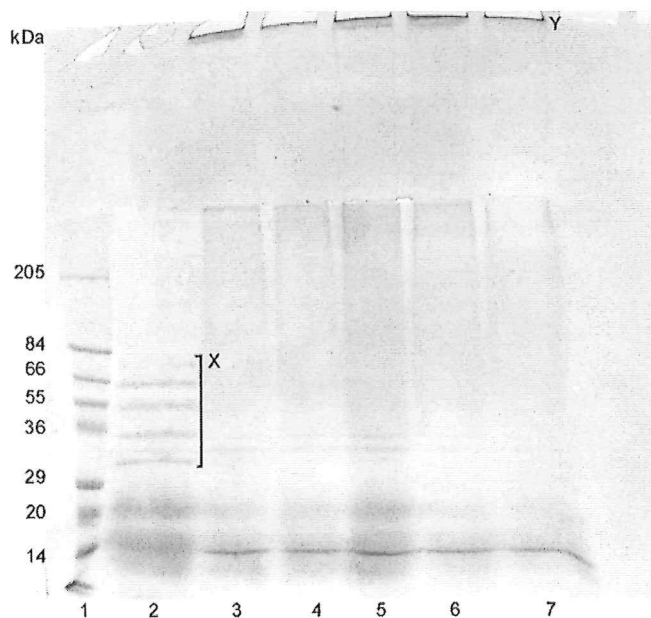


Figure 2.19: SDS-PAGE of croissant gliadins incubated with TGA *in vitro*. **Lane 1:** Sigma marker; **Lane 2:** Control; **Lane 3:** incubation time 5 minutes; **Lane 4:** incubation time 15 minutes; **Lane 5:** incubation time 30 minutes; **Lane 6:** incubation time 1 hour; **Lane 7:** incubation time 24 hours.

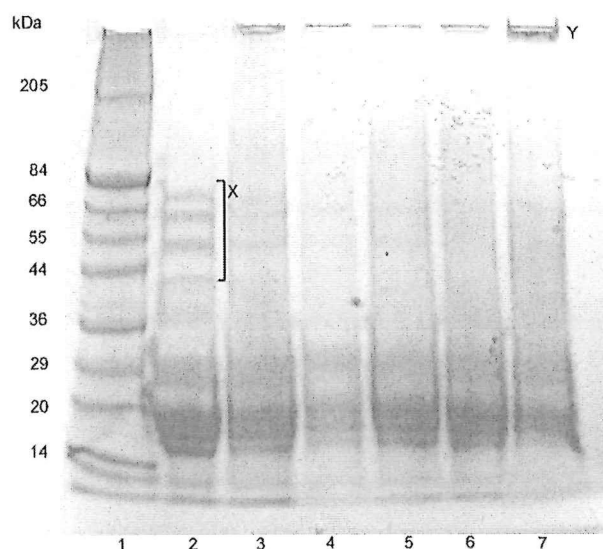


Figure 2.20: Reactive groups on gliadins in a dough network are not available for crosslinking by TGA. Once extracted however, these groups are readily crosslinked.

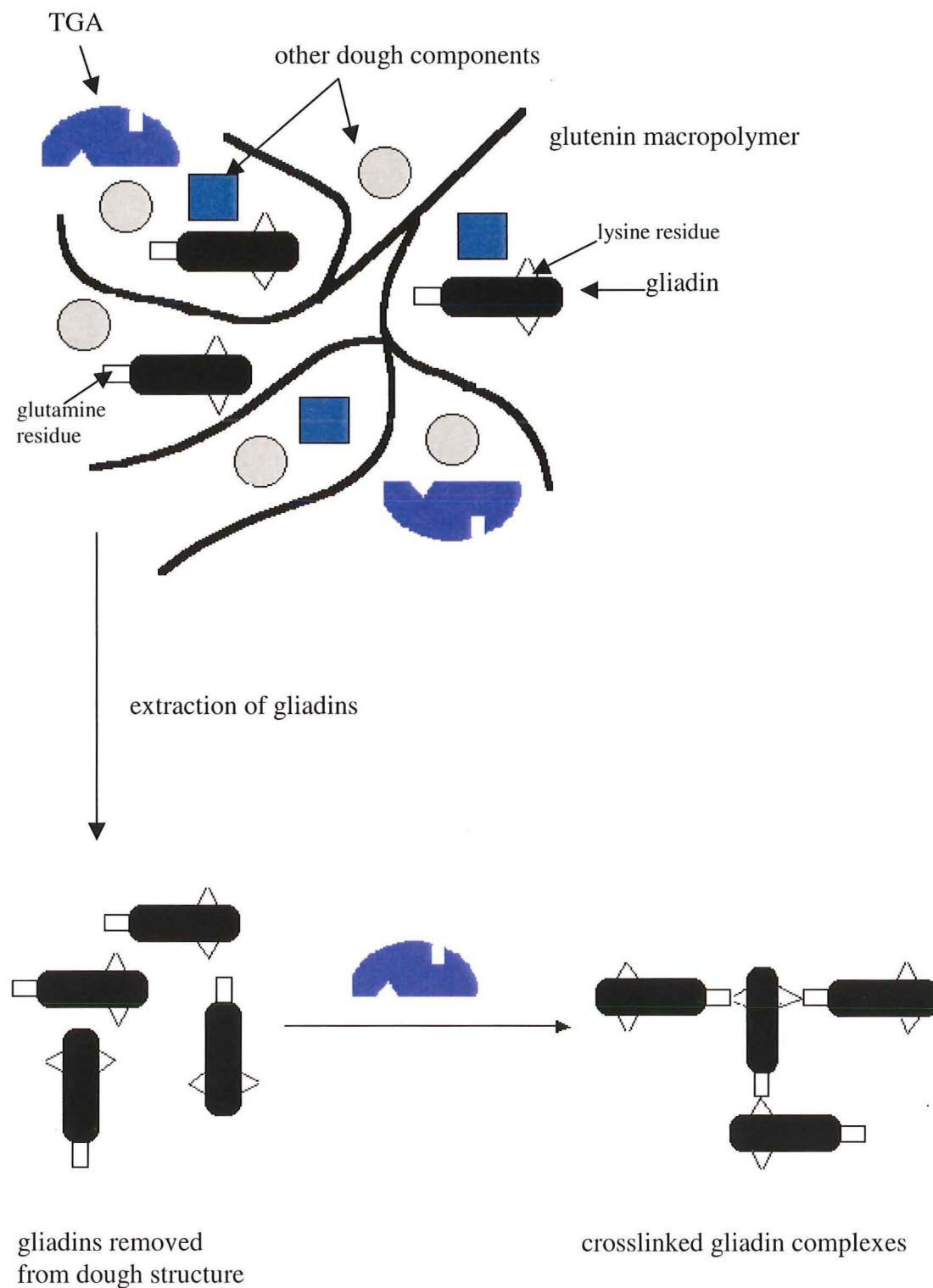


Figure 2.21: SDS-PAGE of bread SDS-soluble glutenins incubated with TGA *in vitro*. **Lane 1:** Sigma marker; **Lane 2:** Control; **Lane 3:** incubation time 5 minutes; **Lane 4:** incubation time 15 minutes; **Lane 5:** incubation time 30 minutes; **Lane 6:** incubation time 1 hour; **Lane 7:** incubation time 24 hours.

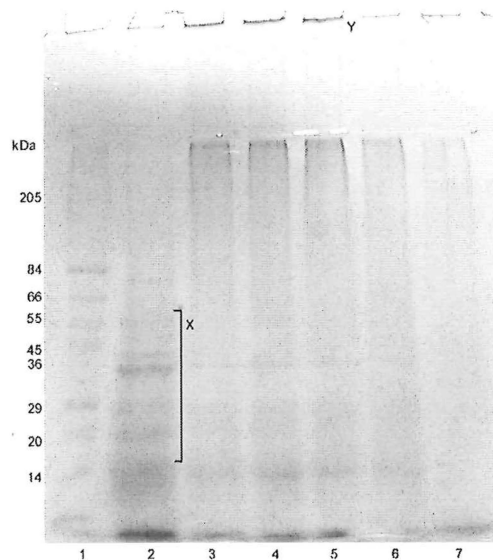


Figure 2.22: SDS-PAGE of croissant SDS-soluble glutenins incubated with TGA *in vitro*. **Lane 1:** Sigma marker; **Lane 2:** Control; **Lane 3:** incubation time 5 minutes; **Lane 4:** incubation time 15 minutes; **Lane 5:** incubation time 30 minutes; **Lane 6:** incubation time 1 hour; **Lane 7:** incubation time 24 hours.

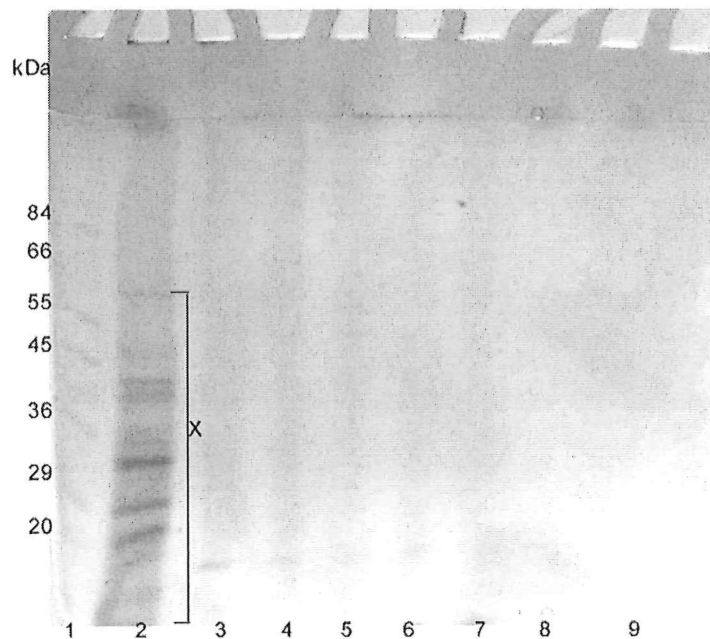


Figure 2.23: SDS-PAGE of bread SDS-insoluble glutenins incubated with TGA *in vitro*. **Lane 1:** Sigma marker; **Lane 2:** Control; **Lane 3:** incubation time 5 minutes; **Lane 4:** incubation time 15 minutes; **Lane 5:** incubation time 30 minutes; **Lane 6:** incubation time 1 hour; **Lane 7:** incubation time 24 hours.

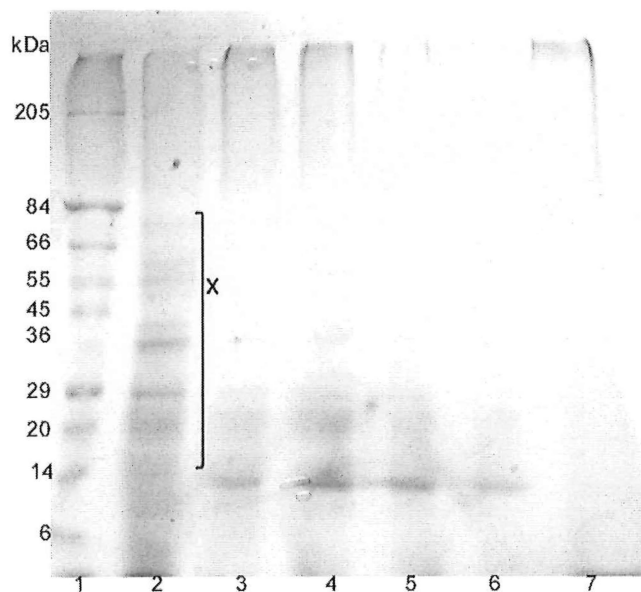
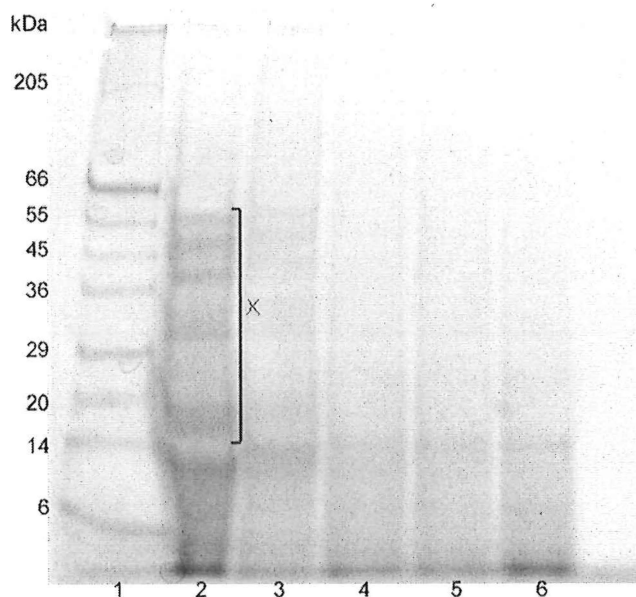


Figure 2.24: SDS-PAGE of croissant SDS-insoluble glutenins incubated with TGA *in vitro*. **Lane 1:** Sigma marker; **Lane 2:** Control; **Lane 3:** incubation time 5 minutes; **Lane 4:** incubation time 15 minutes; **Lane 5:** incubation time 30 minutes; **Lane 6:** incubation time 1 hour; **Lane 7:** incubation time 24 hours.



2.6.3 SDS-soluble glutenins

Figures 2.21 and 2.22 demonstrate the SDS-soluble glutenins from bread and croissants with TGA over time of incubation. The bands labelled X in lane 2 of both figures have been removed in lanes 3-7. Some smearing, indicating the occurrence of crosslinking can be seen in lanes 3-7 of figure 2.21.

2.6.4 SDS-insoluble glutenins

The SDS-insoluble glutenin extracts showed rapid and complete band removal and polymer formation, as expected. Figures 2.23 and 2.24 demonstrate the SDS-soluble glutenins from bread and croissants with TGA over time of incubation. As with the other fractions, the bands labelled X in lane 2 of both figures have been removed in lanes 3-7.

While this confirmed the *in situ* finding that these large proteins met the requirements for crosslinking by TGA, the effect was not more pronounced than in the other fractions. This is consistent with the suggestion of Scardone et al (2000), that whereas within flour the glutenins are more exposed than gliadins, this difference practically disappears upon extraction.

Thus it appears that extraction results in all the different protein groups serving equally well as substrates. These studies have demonstrated that in considering the reactions of food proteins, it is essential to take into account the environment in which they occur.

2.7 Summary

This research has identified the specific proteins of wheat-based dough that are crosslinked by TGA, which has been shown to significantly improve the quality of baked goods. Thus the crosslinking reaction by TGA, known to polymerise proteins in other foods, has now been demonstrated to take place in wheat proteins, *in situ*.

Several findings were made, the most outstanding of which was that the TGA-catalysed reaction within the dough was immediate and irreversible, apparently occurring while the doughs were still in the mixer. No further changes were seen upon

the introduction of variables such as time of proofing and increased enzyme concentration.

While all fractions except the gliadins were crosslinked by TGA, the largest effect of all was observed on the SDS-insoluble glutenin fraction, which consists mostly of high molecular weight glutenins. It seems that these large polymeric proteins were crosslinked into extremely large aggregates, thereby enormously enhancing their role in dough strength and product volume, particularly in croissants. The crosslinking of the albumin/globulin fraction, although less pronounced, may have contributed to the overall improver effect especially with regard to crumb texture and water absorption.

Studies conducted *in vitro* showed that all the protein fractions, including the gliadins, could be crosslinked by TGA. The difference in the TGA response of the gliadins within and outside the dough was an unexpected finding. It has been proposed that both the presence of the reactive groups and conformation of the proteins within the dough are important in determining the extent of crosslinking by TGA.

These findings are likely to be very valuable for the baking industry, which constantly seeks to find ways of improving product quality. As crosslinks are introduced by TGA via a non-oxidative mechanism, this enzyme could potentially replace costly procedures associated with traditional oxidative flour improvers. The immediate nature of the TGA reaction as well as its stability under varying processing conditions add to the advantages of its use in baked products.

In *Chapter Three*, the impact of a third variable increasingly encountered in baked products, namely long-term frozen storage, is considered in relation to the TGA reaction.

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Chapter Three

THE EFFECT OF TRANSGLUTAMINASE ON FROZEN CROISSANT DOUGHS

3.1 Background

3.1.1 Production of frozen doughs

Breads, as well as croissants and other pastries that have been freshly baked, are among the best selling items in the food industry (Schroeder, 1999). As a consequence, one segment of the baking industry that has developed during the past several years is that of frozen doughs, in which doughs prepared at a central, high-production facility are frozen, distributed and subsequently baked for the consumer (Kulp, 1995; Spooner, 1997). The use of frozen doughs saves time, space and equipment costs, thus allowing the economic production of a variety of high value baked products for local consumption or export.

As a food processing operation, freezing is conducted for one of two reasons: to cause desirable textural changes, as in frozen dairy products; and/or to improve storage stability (Matz, 1989). While baked products are often frozen to prevent staling (Cauvain, 1998 a), doughs are increasingly being frozen to maintain quality over long time periods prior to baking (Inoue and Bushuk 1992; Stauffer, 1993).

3.1.2 Changes to bread dough caused by freezing

When doughs are frozen for long periods of time, quality deterioration can occur, resulting in decreased volume in products such as bread and croissants, and longer proofing time. To obtain good quality from frozen doughs it is often necessary to modify the product formulation and processing conditions. Generally, the stronger the flour, the better will be the ability of the product to survive long periods in frozen storage (Wang and Ponte, 1994). In New Zealand, it has been found that strong flours from wheats such as cultivar Endeavour perform well under freezing conditions

(Newberry, 1995). The use of dough conditioners and flour improvers also helps to reduce dough weakening. Ascorbic acid, in particular, is often used in dough formulations for this purpose (Nakamura and Kurata, 1997). The development of raw materials that are more resistant to the processes of freezing and thawing has been suggested as the first stage to reducing textural damage in frozen foods (Kennedy, 2000). However, the use of enzymatic flour improvers may be a simpler alternative to preserve quality parameters in frozen goods.

3.1.3 Relationship of frozen dough quality to protein structure

Two factors are considered to be mainly responsible for the deterioration in the quality of frozen doughs. These are (1) the loss of yeast activity and (2) the formation of ice crystals which damages the gluten network (Inoue et al, 1994). Much effort has been made to understand the role of yeast death during freezing (Havet et al, 2000; Wolt and D'Appolonia, 1984 a, b; Bruinsma and Giesenschlag, 1984; Gelinas et al, 1994). It is thought that the effect is due, in part, to a change in yeast interactions with constituent starches and partly to the release of reducing substances such as glutathione by dying yeast cells, which weakens the dough by breaking disulfide crosslinks between the gluten proteins (Ribotta et al, 2001).

Studies addressing the weakening of the gluten matrix under freezing conditions include those of Lu and Grant (1999) and Ribotta et al (2001). In the study of Lu and Grant (1999), the contributions of starch and the wheat flour proteins (water-solubles, gliadins and glutenins) to frozen dough quality were examined. Using two flours of diverse baking quality, the fractions from the poor quality flour were progressively replaced by the corresponding fraction from the strong flour, with subsequent analysis of freezing and baking performance. Whilst reconstituted flours performed significantly better overall, it was clear that the best results were obtained by replacement of the glutenin fraction. When the entire glutenin fraction was replaced in these reconstituted frozen doughs, the previously weak flour produced greatly increased loaf volume and decreased proofing time. The effects of the gliadin and starch fractions on frozen dough quality were also significant, however the results were not as definitive as those observed for the glutenin fraction. The effect of substitution of the water-soluble fraction, consisting of albumins and globulins as well as starch, was small, but positive.

More recently, Ribotta et al (2001) examined the effect of freezing on bread dough quality. Analyses of protein extracts by multi-stacking SDS-PAGE and densitometry showed that the deterioration in quality upon freezing was related to a depolymerisation of the glutenin polymers. Specifically, the proportion of glutenin subunits of high molecular weight decreased on freezing and this depolymerisation increased with storage time at -18°C .

Significant changes in the glutenin fractions were also reported by Inoue et al (1994), in their study of dough weakening over 70 days in frozen storage. SDS-PAGE analyses showed that while albumin and globulin and gliadin fractions showed only minor differences upon removal from frozen storage, noticeable differences were evident in the glutenin pattern. Specifically, several high molecular weight bands were absent, indicating that the structure of the glutenin protein is definitely altered by repeated freezing and thawing. These researchers also suggested that the small changes to the water-soluble protein may be more significant to the changes in the gluten network than the release of glutathione by yeast cells.

In our investigation of the effect of added TGA in frozen croissants, the albumin/globulin, gliadin and SDS-soluble and insoluble glutenin fractions were extracted from the croissants following fat extraction by the Soxhlet method. The impact of TGA on the individual fractions was interpreted in the context of the studies summarised above.

3.1.4 The freezing trials

Previous investigations had indicated that croissants and pastries prepared with TGA performed better under frozen storage conditions than controls without the added enzyme (Gerrard et al, 2000). In these former trials investigating the addition of TGA to yeasted croissants, the improver effect was maintained for up to 90 days in the freezer at -20°C . The dosages in these trials were 2500 p.p.m. and 5000 p.p.m., due to the low percentage of enzyme (20%) in the commercial preparation. These trials found that treated doughs were stiffer and less extensible than controls and that the treated doughs were in excellent condition upon defrosting in comparison to controls, which showed visible crust damage and fat leakage (figures 3.1 and 3.2). The addition of

TGA eliminated the need for the addition of gluten into the formulation to improve baking quality and storage stability.

We therefore undertook further trials to explore these effects and to elucidate the nature of the improvements at the molecular level. This chapter describes results from two identical trials in which yeasted croissants prepared with and without TGA were frozen for 90 days at -20°C . Croissant samples were removed from the freezer at specified time intervals and analysed for changes both macroscopically and at the molecular level by SDS-PAGE and HPLC. Comparisons were also made between croissant samples submerged in liquid nitrogen directly from the mixer and those proofed for 50 minutes at 23°C prior to freezing and analysis.

The time intervals in the freezer were chosen to represent industrially relevant periods of storage. These were: day 0 (unfrozen), day 1 (1 day in frozen storage) and days 7, 30 and 90 (7, 30 and 90 days in frozen storage).

3.2 Physical changes to croissants over time in frozen storage

In our present study, the effect of adding TGA to the croissant formulation was evident immediately upon preparation of the doughs. TGA-treated doughs were considerably stronger than controls and appeared able to withstand a greater level of stretching without showing signs of cracking. Replicates among the TGA doughs also displayed more consistency in shape than controls.

Further differences became clear when the croissant dough samples were removed from the freezer at the various time intervals. As frozen doughs are thawed prior to baking, the proofing of representative samples from the different time intervals in this study enabled a comparison of the condition of the proofed croissants with and without TGA. Upon proofing, controls generally remained relatively flat whereas TGA-treated croissants displayed a more rounded shape.

Figure 3.1: Cross section through croissants baked after one month in frozen storage, resulting from a former trial; from left to right: TGA 2500 p.p.m + 2.5% gluten; control; TGA 5000 p.p.m. The TGA croissants are larger and better formed. Picture taken from Gerrard et al (1997)

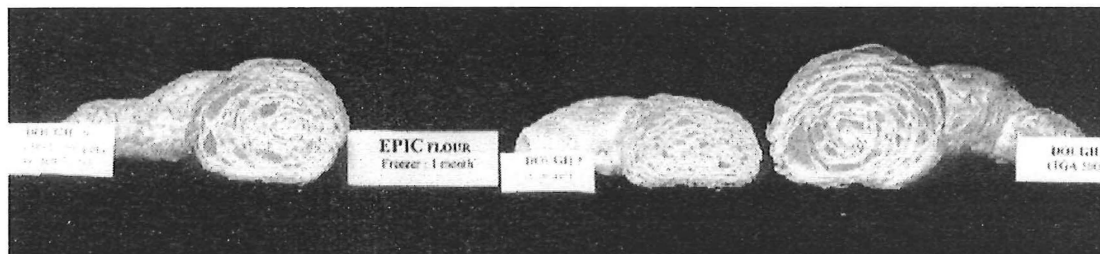


Figure 3.2: Whole croissants baked after one month in frozen storage, resulting from a previous trial; from left to right: TGA 2500 p.p.m + 2.5% gluten; control; TGA 5000 p.p.m. Croissant with TGA 5000 p.p.m (far right) shows better size and shape than the control or TGA 2500 p.p.m + 2.5% gluten. Picture taken from Gerrard et al (1997).

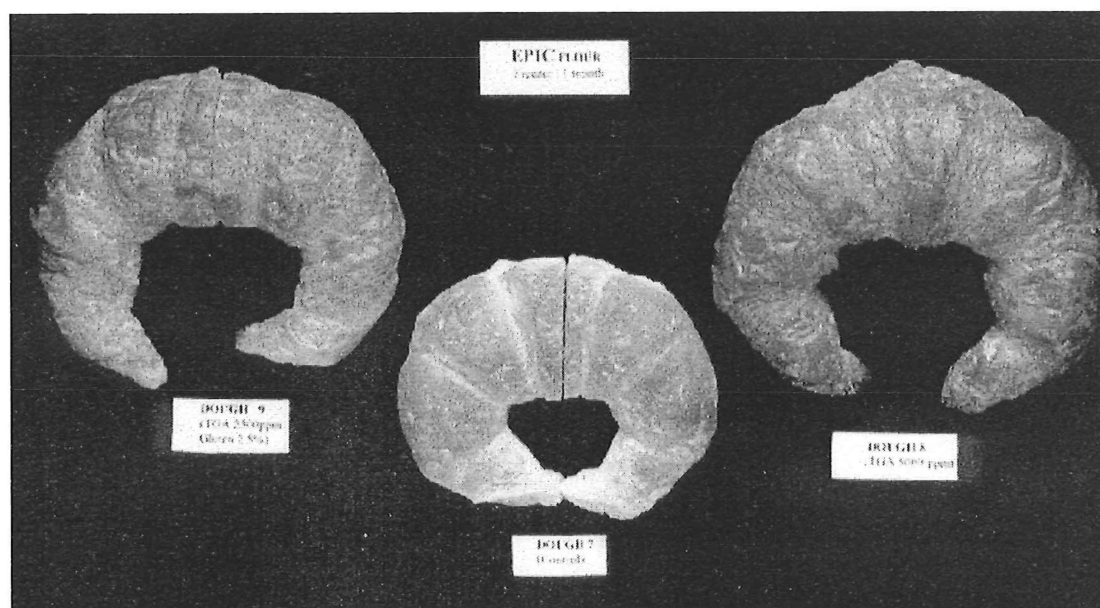


Figure 3.3 (a): Whole croissants, with (upper) and without (lower) TGA, baked following 1 week in the freezer.

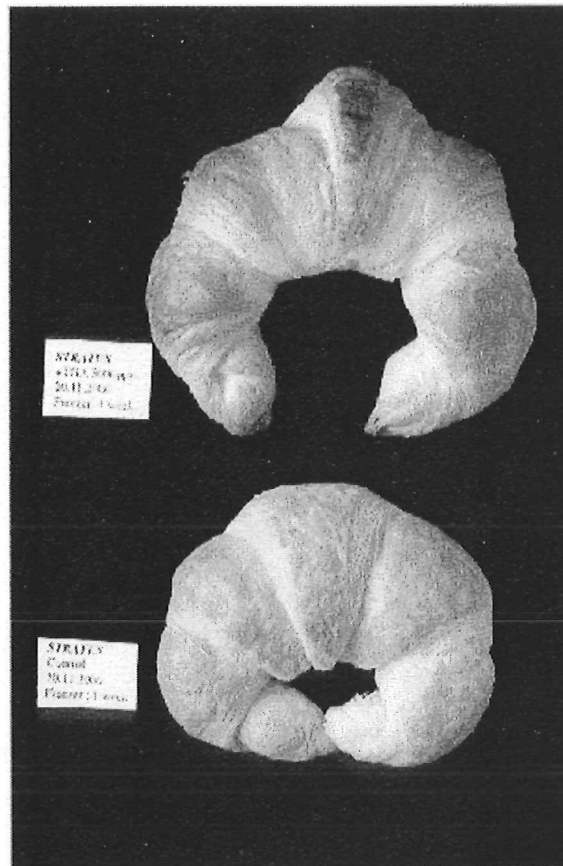
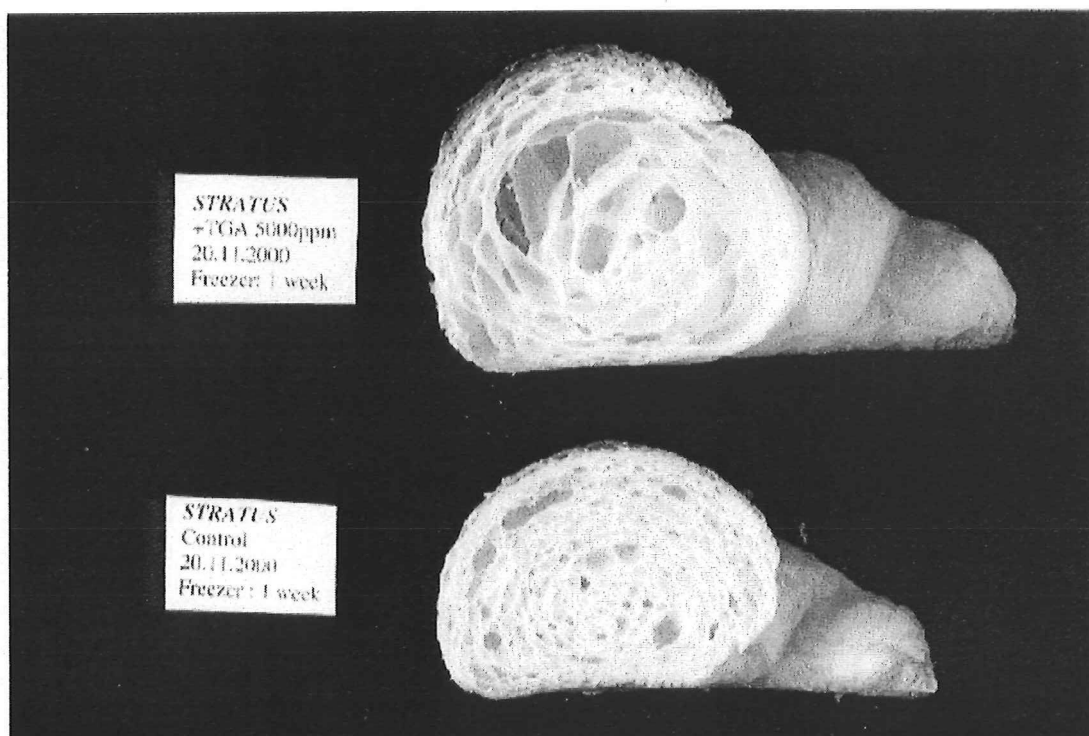


Figure 3.3 (b): Cross section through croissants, with (upper) and without (lower) TGA, baked following freezing 1 week in the freezer.



This effect became more obvious when the croissants were baked (figures 3.3 a and b). When baked, the control croissant samples showed a flattening of the crust and leakage of fat giving a blotched appearance whereas TGA croissants displayed a rounded shape with a smooth appearance. In addition, controls had a gas cell structure consisting of very small cells distributed unevenly, whereas TGA-treated ones showed large gas cells in a desirable honeycomb pattern. Whilst some changes in this pattern took place over the 90 day period, the TGA-treated croissants showed less variation in gas cell pattern than the corresponding controls. It was thought that these changes were due to the effect of TGA on the dough proteins. Thus, the general observation was one of gradual deterioration in the controls, while TGA croissants maintained their improved qualities throughout the three months.

These results were entirely consistent with the results of previous trials (Gerrard et al, 2000) which showed an approximately 60% increase in croissant volume with TGA treatment. However, it became clear that the TGA croissants did undergo some change over the freezing time, and this was evident mainly in the crumb patterns. Although parameters such as volume and general outer appearance remained unchanged once the initial TGA response had taken place, examination of the cross section of these baked samples showed that during freezing, the gas cells became uneven, causing the crumb pattern to lose its uniformity. This effect was similar to that in over-oxidised doughs, as described by Cauvain (1998 b).

A comparison of the baked croissants from the day 0 group is shown in figures 3.4 (a and b). TGA-treated samples were much larger and had a better overall shape, than controls. TGA samples also displayed significantly better definition of the layers than the controls.

After one week in frozen storage, the controls showed a slight decrease in volume and blistering of the crust whereas TGA croissants maintained their size, shape and crust texture. With increasing storage time, further deterioration in volume took place in the controls, accompanied with the gas cells decreasing in size (figures 3.5 a).

Figure 3.4 (a): Cross section through typical control croissants, day 0.

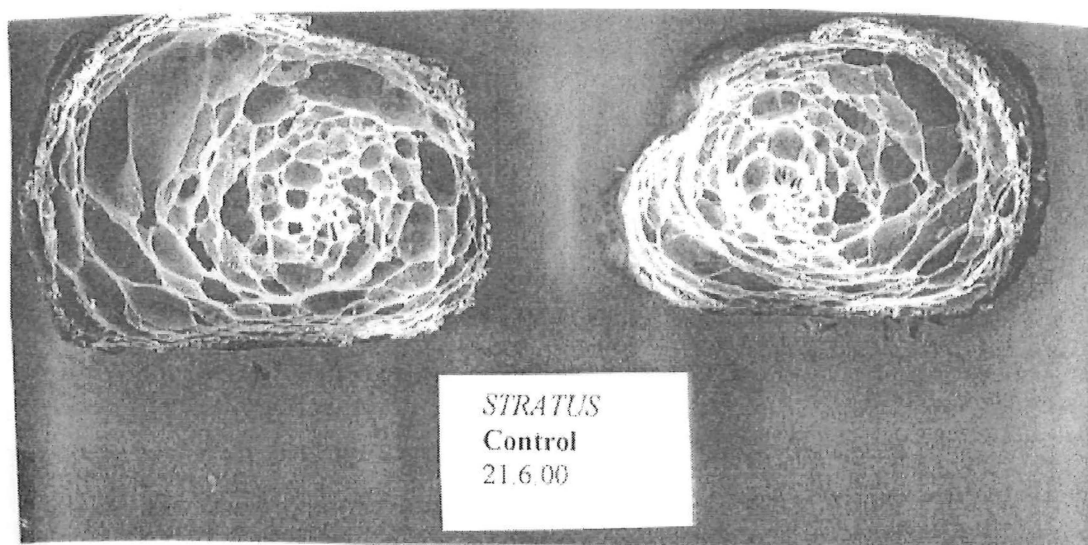


Figure 3.4 (b): Cross section through typical TGA-treated croissants, day 0.

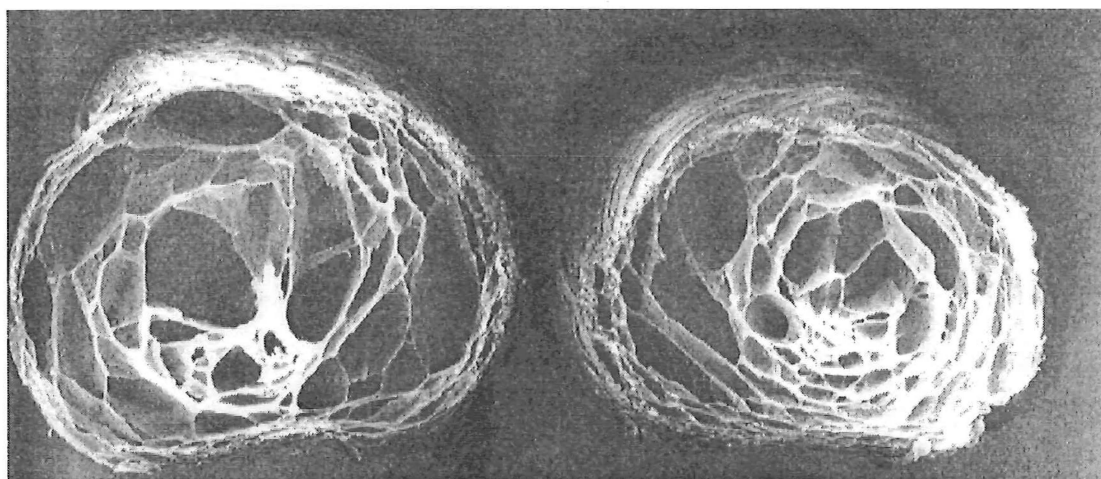


Figure 3.5 (a): Cross section through typical control croissants, day 30.

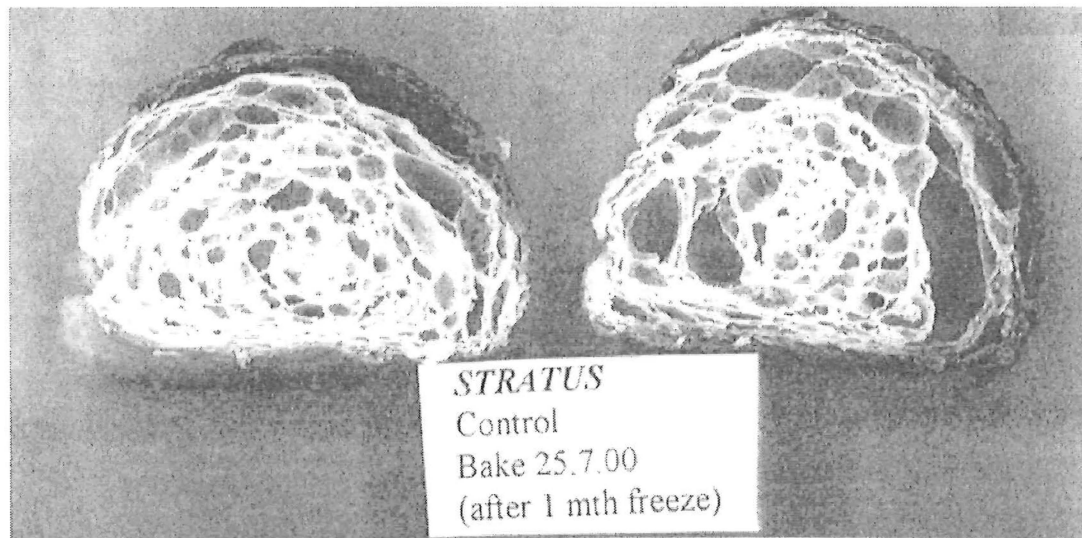


Figure 3.5 (b): Cross section through typical TGA-treated croissants, day 30.

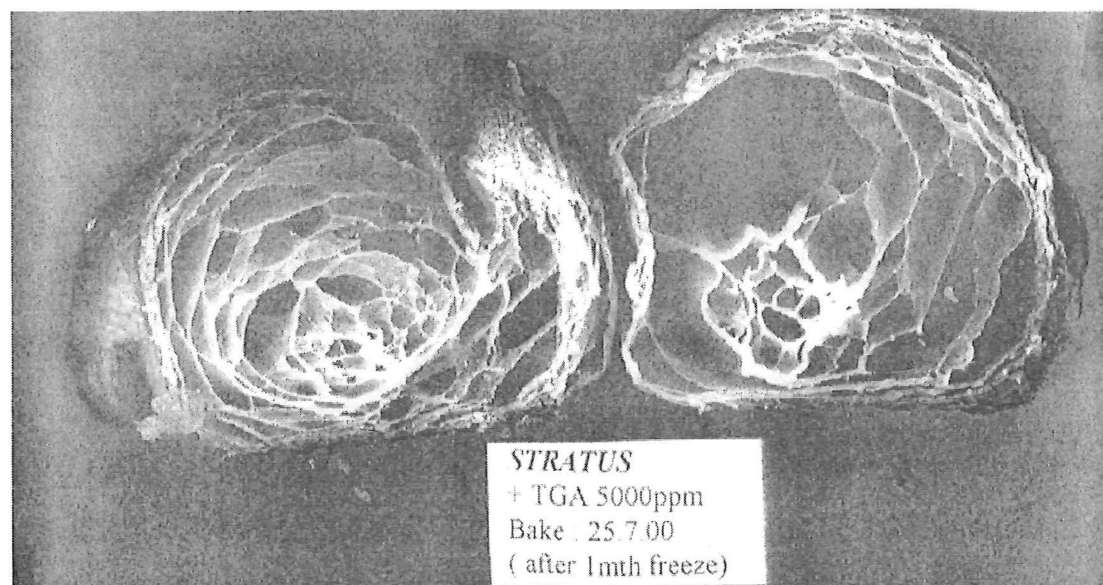


Figure 3.6 (a): Cross section through typical control croissants, day 90.

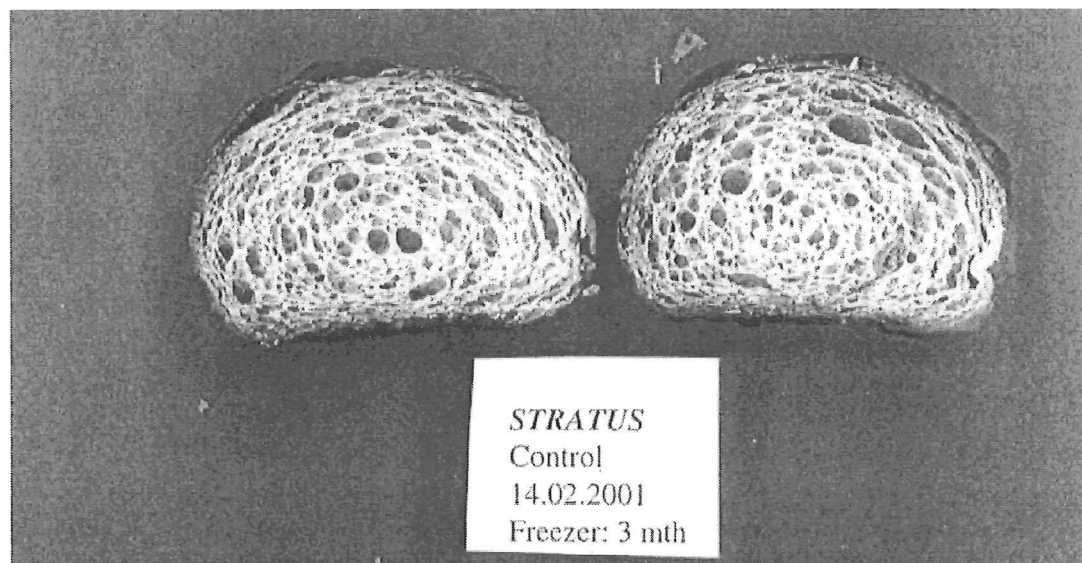
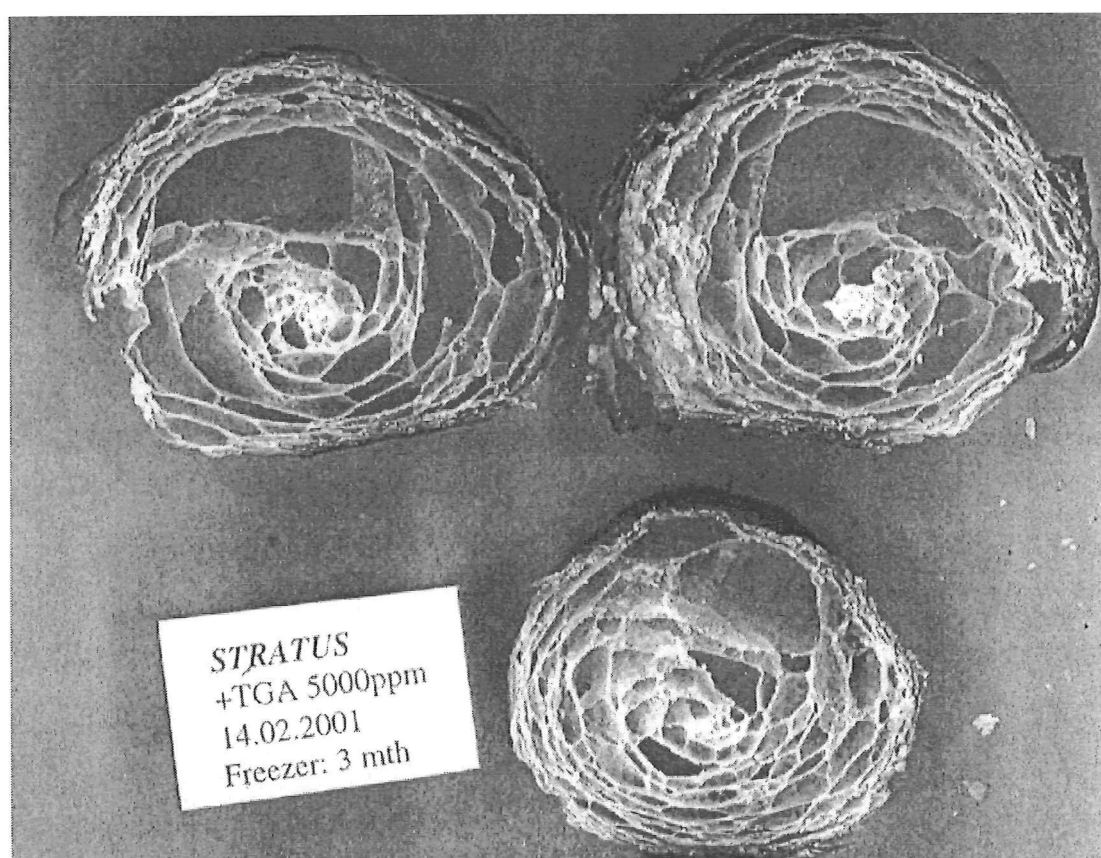


Figure 3.6 (b): Cross section through typical TGA-treated croissants, day 90.



In contrast, the TGA croissants continued to stay in good overall condition, with the only change being evident in the gas cells becoming uneven (figure 3.6 a and b). The large spaces seen in the treated croissants are less than optimal, indicating the requirement for further optimisation of the processing conditions for doughs being prepared for freezing.

Representative samples from different storage periods are shown in the figures 3.4 – 3.6.

The main observation in both trials was one of TGA causing significant improvements in quality characteristics of the frozen croissants, which were maintained over the time of storage. The remainder of this chapter therefore seeks to explain these macroscopic properties by examining molecular changes to dough protein fractions as determined by SDS-PAGE and SE-HPLC.

3.3 SDS-PAGE analysis of croissant protein extracts

Multiple analyses were carried out for the protein samples from each freezing time interval. The extracts were run on 8% polyacrylamide gels under reducing conditions and the resulting protein profiles were compared to analyse differences between TGA-treated samples and the corresponding controls. The first finding was that the proofed samples yielded identical results to those placed in liquid nitrogen upon removal from the freezer. The lack of change from the initial TGA effect on the proteins during proofing is a significant finding, as it establishes that the enzyme causes no further changes during this period. Therefore, the results of proofed and non-proofed samples will be discussed simultaneously below, for each protein group.

3.3.1 Albumins and globulins

Figure 3.7 shows a typical gel from SDS-PAGE of the albumin and globulin fraction. Smearing was evident in the lanes corresponding to TGA-treated croissants from each time interval (lanes 2, 4, 6), accompanied by aggregated material at the top of these lanes, indicating the formation of crosslinked polymers too large to enter the gel.

While the addition of TGA caused clear aggregation of albumins and globulins, no individual bands were removed by the enzyme. Neither was there any change in the control samples over freezing time. This effect appeared to be maintained over the 90 day period. Overall, the effect of TGA on the albumins and globulins was one of immediate crosslinking of some of the protein, as evidenced by the formation of aggregated material at the top of the TGA lanes. The fact that there was no obvious removal of individual bands in this fraction suggests that the effect of TGA is non-specific in the albumins and globulins. It is clear that any benefits due to the crosslinking of these small proteins by TGA, are maintained throughout freezing time.

These results are compatible with those reported by Lu and Grant (1999) who found a small but significant contribution from the water soluble fraction of a strong flour, when this fraction was used to replace the water-soluble fraction of a weak flour. Changes to the water-soluble protein group from frozen doughs were also reported by Inoue et al (1994). It was suggested by Lu and Grant (1999) that the resulting changes to freezing performance was related mainly to changes in the interactions between flour components, caused by alterations to the fractions. If so, the crosslinking of some of the albumin/globulin proteins into larger and more stable forms may prevent the deteriorative changes occurring in the freezer. Alternatively, the crosslinking of albumins and globulins by TGA may simply improve the strength and texture of the crumb, which is then maintained successfully over freezing time.

3.3.2 Gliadins

A typical gel of gliadin fractions from control and TGA-treated frozen doughs is shown in figure 3.8. Consistent with our previous observations with gliadins *in situ*, the addition of TGA had no major effect on this protein group, other than a slight fading of the band at 66 kDa. Moreover, no additional changes were evident over time in frozen storage. While the substitution of gliadins from strong flours into weak ones improved freezing performance (Lu and Grant, 1999), the gliadin profiles of control samples in our study showed no obvious changes over time in the freezer. As such, the non-responsiveness of the gliadins to the presence of TGA appears inconsequential to freezer-induced changes.

Figure 3.7: SDS-PAGE of albumins and globulins from TGA-treated and control croissants over 90 days in frozen storage. **Lane 1:** Sigma marker; **Lane 2:** TGA-treated albumins and globulins, day 0; **Lane 3:** control albumins and globulins, day 0; **Lane 4:** TGA-treated albumins and globulins, day 30; **Lane 5:** control albumins and globulins, day 30; **Lane 6:** TGA-treated albumins and globulins, day 90; **Lane 7:** control albumins and globulins, day 90.

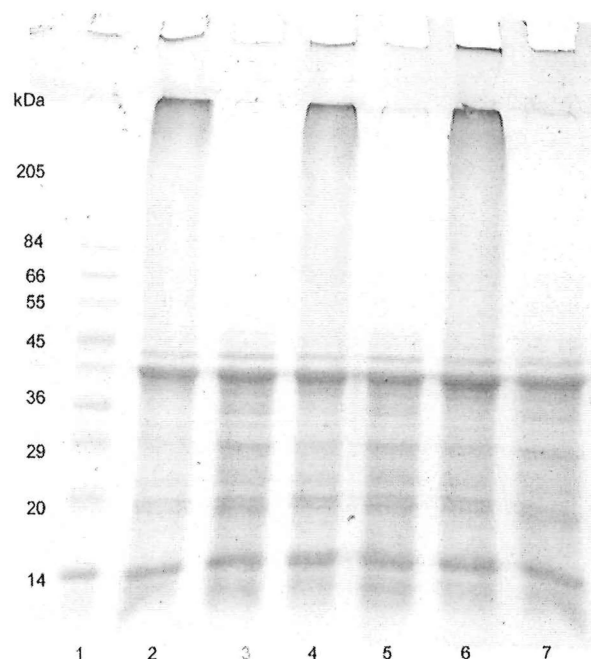


Figure 3.8: SDS-PAGE of gliadins from TGA-treated and control croissants over 90 days in frozen storage. **Lane 1:** Sigma marker; **Lane 2:** TGA-treated gliadins, day 0; **Lane 3:** control gliadins, day 0; **Lane 4:** TGA-treated gliadins, day 30; **Lane 5:** control gliadins, day 30; **Lane 6:** TGA-treated gliadins, day 90; **Lane 7:** control gliadins, day 90.

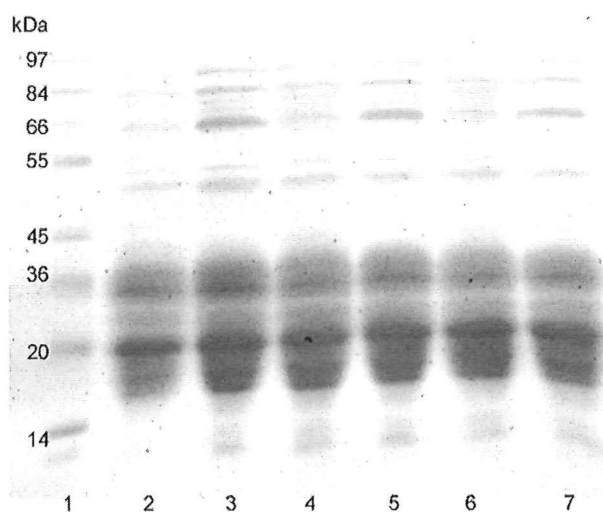


Figure 3.9: SDS-PAGE of SDS-soluble glutenins from TGA-treated and control croissants over 90 days in frozen storage. **Lane 1:** Sigma marker; **Lane 2:** TGA-treated SDS-soluble glutenins, day 0; **Lane 3:** control SDS-soluble glutenins, day 0; **Lane 4:** TGA-treated SDS-soluble glutenins, day 30; **Lane 5:** control SDS-soluble glutenins, day 30; **Lane 6:** TGA-treated SDS-soluble glutenins, day 90; **Lane 7:** control SDS-soluble glutenins, day 90.

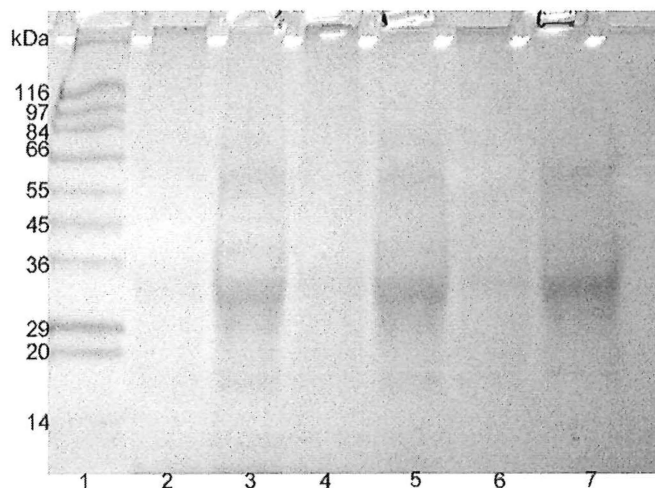
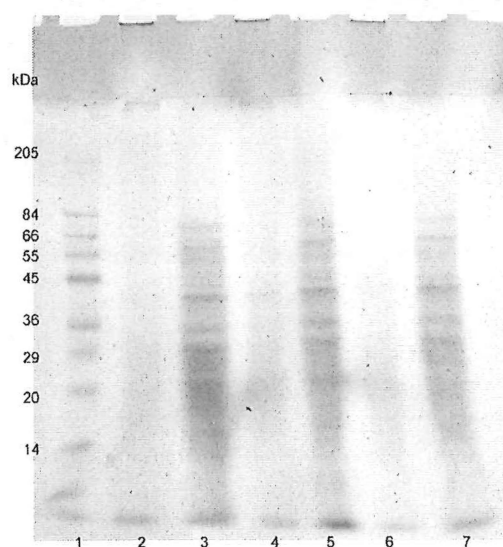


Figure 3.10: SDS-PAGE of SDS-insoluble glutenins from TGA-treated and control croissants over 90 days in frozen storage. **Lane 1:** Sigma marker; **Lane 2:** TGA-treated SDS-insoluble glutenins, day 0; **Lane 3:** control SDS-insoluble glutenins, day 0; **Lane 4:** TGA-treated SDS-insoluble glutenins, day 30; **Lane 5:** control SDS-insoluble glutenins, day 30; **Lane 6:** TGA-treated SDS-insoluble glutenins, day 90; **Lane 7:** control SDS-insoluble glutenins, day 90.



3.3.3 SDS-soluble glutenins

Although in the SDS-soluble glutenin fraction the differences caused by TGA were rather variable, there was definite evidence of crosslinked aggregates at the top of most TGA lanes (figure 3.9). The absence of such material in other TGA lanes of this fraction indicates the formation of polymers too large to enter the gels. The significant feature, as with the other protein fractions was the obvious maintenance of the initial effect throughout freezing time.

3.3.4 SDS-insoluble glutenins

As with our studies described in *Chapter Two*, the most dramatic effect of TGA treatment was evident in the SDS-insoluble glutenin fraction. Once again, almost complete disappearance of bands was observed from day 0, indicating the immediate crosslinking of these large proteins into very much larger complexes. Many of the gels of this fraction showed polymeric material in the TGA lanes, but as with the SDS-soluble fraction, these products were most likely to be too large to enter the gel analyses. As with other fractions, no change from the initial effect was observed during the freezing period, further confirming that the crosslinking effect of TGA is stable under these conditions. A typical gel of SDS-insoluble glutenins is shown in figure 3.10.

The dramatic crosslinking response of the SDS-insoluble glutenins to TGA, and the stability of this effect under freezing conditions are most exciting, particularly in light of reported literature. Of particular relevance is the recent report of Ribotta et al (2001), correlating the decline in freezing and baking performance of frozen bread doughs, to a decrease in the amount of SDS-soluble glutenins on multi-stacking SDS-PAGE and a reduction in high molecular weight glutenin subunits in the resolving gels. They have suggested that during freezing, a depolymerisation of the glutenins takes place, thereby reducing the strength of the gluten matrix. This effect was accompanied by macroscopic differences in the functional properties of frozen bread doughs and the subsequently baked loaves. Among the affected properties were loaf volume, crumb texture and required proofing time, all of which showed deteriorative effects. In addition, the gas cells of frozen doughs were smaller than non-frozen controls.

The effect of freezing on the glutenin polymers has also been reported by other researchers, including Inoue and Bushuk (1992) and Inoue et al (1994). It appears that this is the group of dough proteins most significantly affected by freezing. This is also the group most responsible for flour strength (MacRitchie, 1999). In this context, the use of TGA has obvious advantages to doughs prepared for the purpose of freezing. Our results have consistently demonstrated that the high molecular weight glutenins are the proteins most affected by TGA, resulting in products of, among other benefits, greatly increased dough strength. This strongly suggests that it is the formation of the extremely large complexes that is also responsible for the maintenance of dough quality in the freezer.

In spite of the insolubility of the glutenins of high molecular weight, they are clearly very good substrates for TGA. The reasons for this are explored in *Chapter Five* of this thesis.

3.3.5 SDS-PAGE results: summary

Our analyses of croissant proteins over time in frozen storage have corroborated the results described in *Chapter Two* of this thesis. The main finding was that the effect of TGA on the croissants is immediate and that this effect is maintained throughout time in the freezer. Proofing of the doughs produced no additional effect. It is of great advantage to the baking industry that an enzymatic alternative to chemical improvers is able to provide doughs with the strength required to withstand freezing conditions.

3.4 SE-HPLC analysis of croissant protein extracts

In *Chapter Two*, SE-HPLC was employed to observe changes caused by TGA to the proteins of bread and croissants and biscuits. Analysis of SE-HPLC traces involved calculation of the peak areas, which represented the amount of protein present in a particular group. A decrease in the extractable protein was seen as an indicator of the formation of large, strong complexes, too difficult to extract.

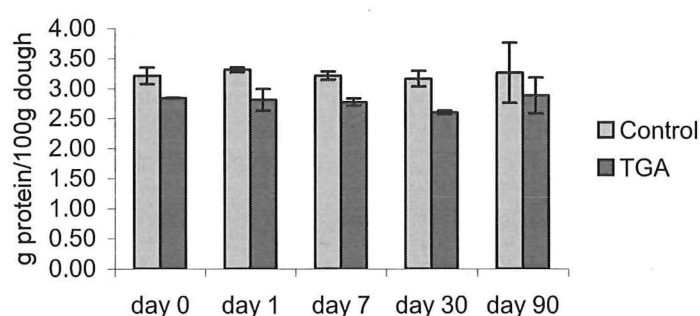
As with the previous work, analyses of dough samples by SDS-PAGE gave useful qualitative results. The presence of polymeric material and especially the smearing in

TGA lanes had demonstrated the crosslinking effect of TGA. Thus in order to obtain results of a quantitative nature, SE-HPLC was applied to the analysis of the proteins from frozen croissants, as described in *Chapter Two*.

3.4.1 Albumins and globulins

A very small, but consistent, drop was seen, for the total proteins extracting in the albumin/globulin fraction, upon treatment with TGA (figure 3.11). This corroborated the results of the SDS-PAGE analyses. Analysis of individual peaks revealed that, interestingly, it is the smaller and intermediate proteins within the albumin fraction that were crosslinked by TGA.

Figure 3.11: Total albumins/globulins from control and TGA-treated croissant doughs in frozen storage up to 3 months. Error bars represent standard error of the mean of 2 samples.



As the nutritionally vital proteins to the growing embryo in the wheat grain, the amino acid composition of the albumin and globulin fraction differs from that of the larger, gluten-forming proteins. Albumins and globulins have significantly more lysine, aspartic acid, alanine, valine and threonine than do the gliadins and glutenins but have less glutamic acid (Dubetz et al, 1979; Lasztity, 1984).

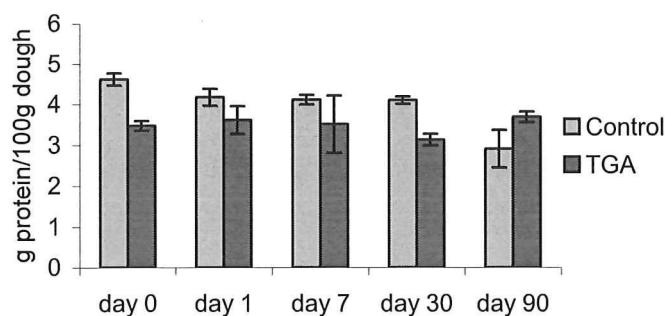
The higher content of lysine, as shown in lysine analyses to be addressed in *Chapter Five*, may play a part in the observed decrease in albumins due to the formation of the ϵ -(γ -glutamyl)lysine crosslink.

While these small proteins are unlikely to participate in the observed great strengthening of croissant dough, it is nevertheless possible that these contribute to improvements in smaller-scale improvements such as crumb texture.

3.4.2 Gliadins

SE-HPLC results of this fraction showed a small drop in extractable protein in some of the time intervals, but an increase in others. While this proved inconclusive, TGA did not appear to be causing a significant effect on the gliadins. This seemed to be consistent with SDS-PAGE results for this group (figure 3.12).

Figure 3.12: Total gliadins from control and TGA-treated croissant doughs in frozen storage up to 3 months. Error bars represent standard error of the mean of 2 samples.



3.4.3 SDS-soluble glutenins

At around 2 g/100 g dry dough, the soluble glutenins remained extractable at a lower concentration than the other protein groups during frozen storage. This is consistent with the SDS-PAGE analyses showing that bands from this fraction were generally fainter than the other fractions (figure 3.13). There was no significant difference in the amount of SDS-soluble glutenin extracted from the treated and control doughs, as measured by SE-HPLC, which was consistent with the generally variable results seen with this fraction on SDS-PAGE.

Figure 3.13: Total SDS-soluble glutenins from control and TGA-treated croissant doughs in frozen storage up to 3 months. Error bars represent standard error of the mean.

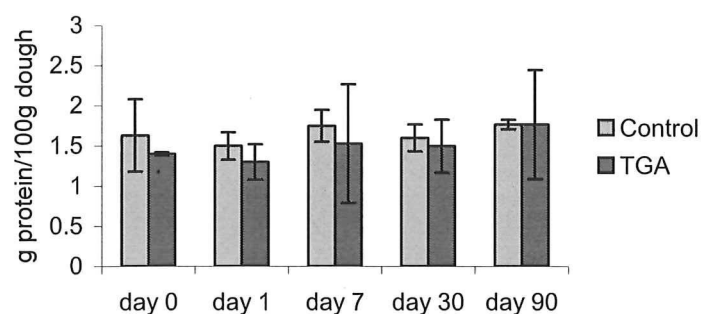
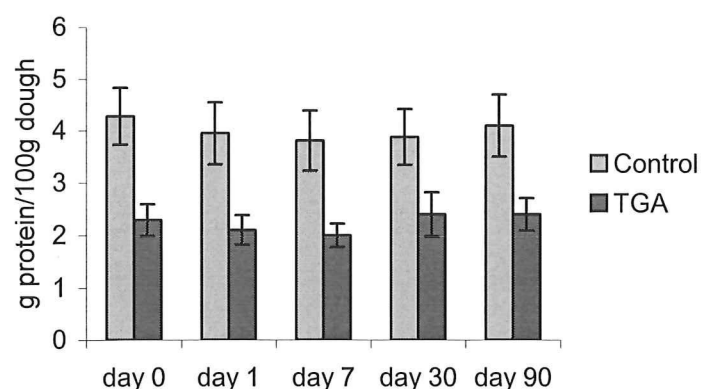


Figure 3.14: Total SDS-insoluble glutenins from control and TGA-treated croissant doughs in frozen storage up to 3 months. Error bars represent standard error of the mean.



3.4.4 SDS-insoluble glutenins

Consistent with our SDS-PAGE results, the immediate and dramatic effect exerted by TGA on the high molecular weight glutenin group, was once again the most outstanding feature in this investigation. While controls were extracted at a total concentration of around 4.5g /100 g dry dough, this was almost halved in the TGA treated samples and maintained during frozen storage (figure 3.14).

The reduction in the extractability of the SDS-insoluble glutenins in the samples treated with TGA indicates that the enzyme is crosslinking them to form polymers of extremely high molecular weight, which are insoluble in SDS even with the use of sonication.

Polymeric aggregates of gluten proteins range in size to well over 10 kDa. On treatment with the reducing agent mercaptoethanol, disulfide bonds are broken, while chaotropic agents such as SDS disrupt hydrophobic bonds between protein chains to facilitate analysis. Thus, although the polymers have been broken down considerably for analysis, it should be appreciated that it is proteins of a very large size that are seemingly preferentially crosslinked by TGA, to form even larger ones, thereby producing doughs with extremely high strength and stability.

3.4.5 SE-HPLC results: summary

The results of the SE-HPLC analysis confirmed the results from the SDS-PAGE analysis. A minimal crosslinking effect was seen in the albumin/globulin fraction, which was maintained over time. Gliadins did not appear greatly affected by TGA treatment, whereas the SDS-soluble glutenins showed some effect. The outstanding feature of the SE-HPLC analysis was once again the response of the SDS-insoluble glutenin fraction, which displayed a dramatic reduction in extractable protein from day 0, which was maintained up to day 90. The crosslinking of this group by TGA is most likely to have been responsible for the improvements in the physical properties of croissants and the maintenance of the improvements over time of freezing.

3.5 Conclusion

The increasing application of freezing technology to baked products has called for the development of raw materials and additives that can withstand long periods at subzero temperatures. The utilisation of TGA in croissant formulations not only yields products of superior quality, but appears to be particularly beneficial to those doughs and pastries prepared for the purpose of freezing.

The means by which TGA generates improvements to frozen doughs appears to be the crosslinking of particular protein groups of wheat flour, which is consistent with the findings described in *Chapter Two* of this research. The response of the different protein groups to TGA treatment followed the same pattern as that observed in the work on bread and croissant doughs. While the albumin and globulin proteins that are high in lysine have demonstrated a small but consistent crosslinking effect by TGA, it

is the crosslinking of the large, SDS-insoluble glutenins of wheat gluten that is most implicated in the great strengthening of the gluten network in the frozen doughs.

With reference to the changes in gas cell patterns of the frozen croissants over the 90 day trials, there appeared to be little evidence to correlate these with TGA induced changes. It may be that factors such as changes to starches in the dough (Cauvain, 1998 a) and the formation of ice crystals (Kennedy, 2000) are responsible for these observations. It has been reported by Cauvain (1998 a) that certain physical changes including staling and crumb firming occurring in frozen bakery products, are related to changes in the crystalline state of the starch present.

Although we found that the operation of freezing had no added effect on the relative distribution of protein groups once the initial reaction had occurred, the initial redistribution upon TGA treatment may exert an influence on how long crumb structure is maintained upon freezing. It is known that a 1:1 ratio of gliadin:glutenin is best for quality in baked products, giving a balance between extensibility and elasticity, and that small changes in these groups result in large differences in functional properties (Gupta et al, 1992). Thus the changes in the relative amounts of gliadin and glutenins due to TGA could explain the observed structural changes.

We have demonstrated that the stable TGA effect is sufficient to greatly improve and maintain the quality of frozen croissants. This is of enormous advantage to the baking industry, especially in light of the need for suitable additives to improve and maintain dough quality during frozen storage.

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Chapter Four

ENZYMATIC CROSSLINKING VIA GLUCOSE OXIDASE

4.1 Introduction

Throughout this research, improvements to the functional properties of baked products have been related to the underlying crosslinking of specific wheat proteins. Although the extent of the formation of the ϵ -(γ -glutamyl)lysine crosslink has been found to occur to varying degrees in different wheat protein fractions, the key element to the transglutaminase-induced improvements in dough and croissant properties appears to be the strengthening of the flour through the formation of enormous polymeric complexes, particularly from the SDS-insoluble glutenins.

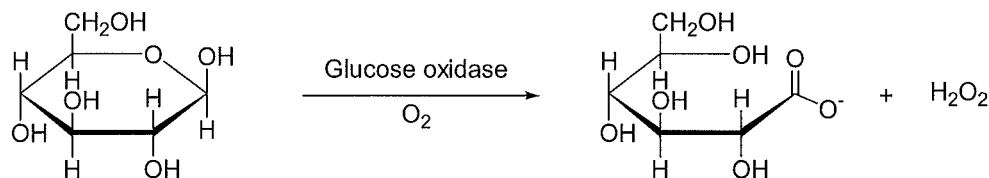
Whilst this work has so far concentrated on introducing crosslinks enzymatically with TGA, this chapter examines the effects of glucose oxidase, an enzyme that is assumed to improve dough properties through the addition of disulfide crosslinks (Vemulapalli and Hoseney, 1998). This crosslinking mechanism is compared to that of TGA, with regard to the proteins altered and the resulting changes in bread and croissants.

4.1.1 Background

Glucose oxidase is derived mainly from fungal sources such as *Aspergillus niger* and *Penicillium amagasakiense* (Barker, 1991; Kalisz et al, 1991). Other organisms that produce it include the actinomycete *Actinobacillus actinomycetum comitans*. Glucose oxidase is used in food processing, in the production of gluconic acid and in the quantitative determination of D-glucose in samples such as food, fermentation products and blood (Kalisz et al, 1991). It has also been used as a dough bleaching agent along with peroxidases and catalases (Gelinas et al, 1998) and occurs as a component of various commercial formulations for flour improvement (Feng, 2001; Polandova et al, 2000).

Glucose oxidase catalyses the conversion of D-glucose to D-gluconic acid by molecular oxygen, which is converted to H_2O_2 (Hillhorst et al, 1999), as illustrated in figure 4.1.

Figure 4.1: Reaction catalysed by glucose oxidase.



The enzyme itself is not an oxidising agent, but the H_2O_2 produced has been correlated to presumed oxidative effects in bread (Liao et al, 1998). This is due to the same effect being achieved with other hexose oxidases that are known to produce H_2O_2 *in situ* (Poulsen and Holstrup, 1998). This effect is thought to involve the formation and rupture of disulfide linkages, which is known to significantly affect functionality, as discussed in *Chapter One* (Okumura et al, 1989; Shewry and Tatham, 1997; Anderson and Ng, 2000).

4.1.2 Purpose for the study of glucose oxidase mediated crosslinking in baked products

In addition to this research with TGA, other work in this laboratory (Gerrard et al, 2002 a, b, c) investigated the addition of introducing non-disulfide crosslinks into baked products using chemicals. Employing the carbonyl compounds glutaraldehyde, glyceraldehyde and formaldehyde, Gerrard et al (2002 a, b, c) showed that, although *in vitro* these compounds could crosslink all the wheat protein fractions except for the gliadins, addition of glutaraldehyde to baked products *in situ* resulted in the crosslinking of only the albumin/globulin fraction. This corresponded with improvements to crumb texture and strength. In contrast, this research has shown that the crosslinking by TGA of mainly the SDS-insoluble fraction resulted in major improvements to dough strength and croissant volume. This suggests that specific functional effects may be attributable to the crosslinking of particular wheat proteins.

Thus, our interest next turned to the addition of crosslinks by a different type of crosslinking enzyme, namely glucose oxidase. It was earlier reported by Vemulapalli and

Hoseney (1998), that H_2O_2 production by glucose oxidase affected mainly the water-soluble (albumin/globulin) fraction, whilst leaving the gluten proteins unaffected. This was based on the observation of a decrease in the thiol content of the water-soluble fraction, and attributed to their oxidation into disulfide linkages. The possibility of other crosslinks being formed was not considered. We therefore sought to examine the crosslinks in the dough proteins, and relate these to changes in functional properties.

Preliminary studies in our laboratory had indicated that both disulfide and non-disulfide crosslinks were formed in bread treated with glucose oxidase (Low, 2000). Therefore, it was of interest to examine the nature of the crosslinks produced, in addition to identifying the wheat protein fractions mostly affected by the crosslinking chemical produced by glucose oxidase. Thus, the three questions addressed in this chapter are:

1. How is the product quality of bread and croissants altered as a result of glucose oxidase treatment?
2. Which protein fractions are most affected by crosslinking?
3. Are the crosslinks produced limited to disulfide bonding?

4.1.3 Comparison of the oxidising effect of chemical improvers with that of the oxidising chemical produced by glucose oxidase

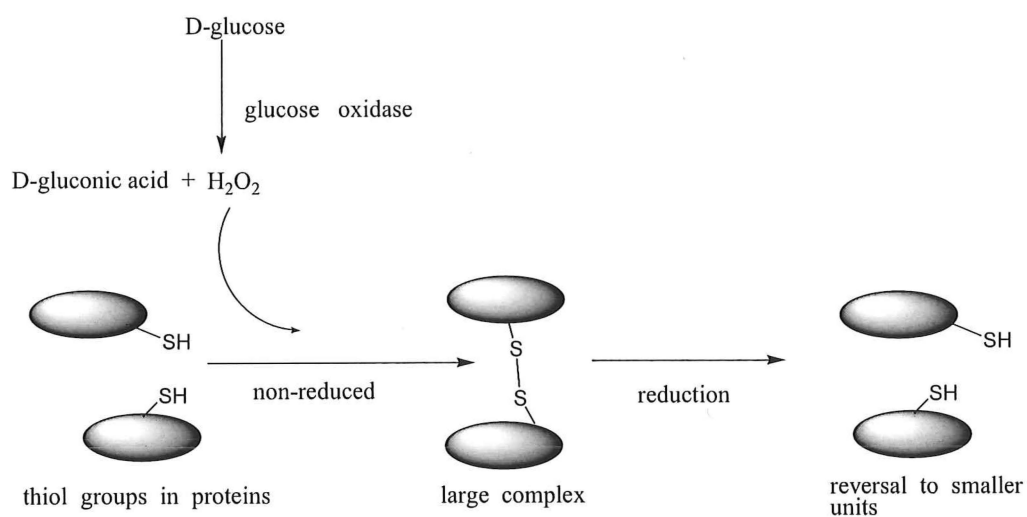
As discussed in *Chapter One*, improver effects have been obtained in bread for many years using oxidising agents. These are assumed to act on the thiol groups of gluten proteins, as evidenced by reversible changes to dough rheology and baking performance (Kulp, 1993; Bekes et al, 1994; Wikstrom and Eliasson, 1998). Detailed studies have been carried out (Veraverbeke et al, 2000 a, b) to show that high molecular weight subunits of glutenin can be polymerised *in vitro* in stepwise oxidation reactions, using KBrO_3 , KIO_3 and H_2O_2 . The molecular mechanisms of the enzyme improvers, however, are generally less well understood than those of chemicals (Si, 1997; Wikstrom and Eliasson, 1998; Vemulapalli et al, 1998).

4.2 Methods for investigation

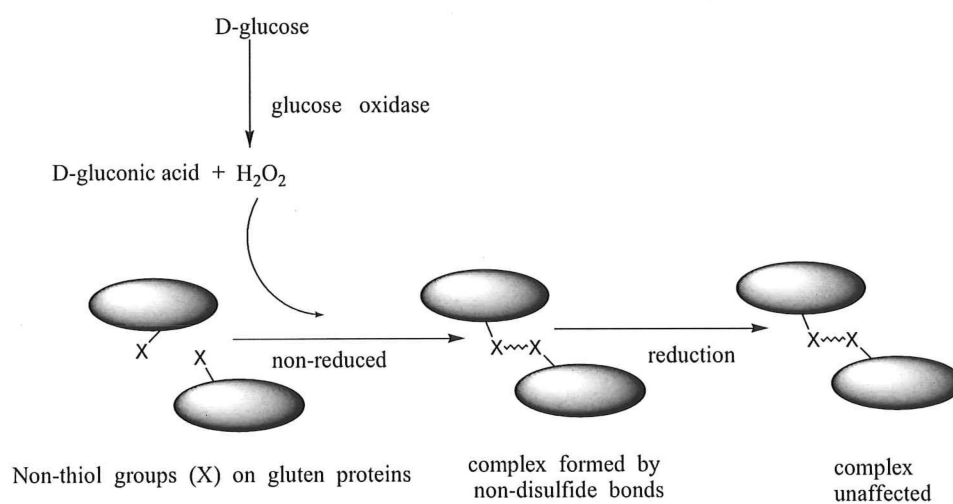
SDS-PAGE and SE-HPLC were once again chosen for analyses of the wheat dough proteins. However, to ascertain more information as to the nature of the crosslinks, SDS-PAGE was conducted under both reducing and non-reducing conditions, since reduction of the samples would break the disulfide bonds, the effect of which we wished to observe. In gluten proteins containing SH groups, oxidation gives rise to large complexes held together by S-S bonds. Reduction of these complexes results in a reversal of this process, yielding smaller units with -SH groups (figure 4.2 a). However, if an oxidising chemical from the glucose oxidase reaction were also responsible for a different type of (non-reducible) crosslink (X), treatment with the reducing agent leaves these large complexes unaffected (figure 4.2 b). Analysis of the treated protein extracts by reducing and non-reducing SDS-PAGE can thus provide clues as to the nature of the crosslinks formed in the treated doughs. The use of reducing and non-reducing SDS-PAGE to detect the extent of disulfide crosslinking has been reported by other researchers, including Anderson and Ng (2000).

Figure 4.2: Schematic description of the possible outcomes of disulfide (a) and non-disulfide (b) polymers under reducing and non-reducing conditions. (Adapted from Low, 2000)

(a) Disulfide polymers



(b) Non-disulfide polymers



4.3 Glucose oxidase effects on bread and croissants

4.3.1 Effect of glucose oxidase treatment on bread properties

To observe the macroscopic effects of glucose oxidase treatment on baked loaves, several bread doughs were prepared, with various treatments. Loaves were prepared in duplicate for each treatment. First, the control doughs were prepared using the standard Crop & Food in-house method, with no improvers. The second treatment contained extra glucose (0.5%), as a verification of the presence of sufficient substrate in dough that had not been supplemented. Treatment three consisted of the addition of 10 p.p.m glucose oxidase to the bread mixture. The fourth treatment was the addition of 10 p.p.m glucose oxidase plus 0.5% glucose. A fifth treatment, namely the addition of a high dose of H_2O_2 to the standard bread mixture was also carried out, in order to definitively detect any molecular effects caused by the enzyme.

Upon baking (figure 4.3), loaves containing extra glucose were found to have a darker crust than those without. This is assumed to be due to Maillard browning caused by the reaction between wheat proteins and the added sugar (Fayle and Gerrard, 2002). Doughs prepared with H_2O_2 were tight and extremely difficult to handle and were half the size of the other loaves. This is likely to be due to the over-oxidising effect, as described in *Chapter One*. Also consistent with over-oxidisation, these doughs had evidence of uneven large gas cells.

Apart from these obvious effects, no significant differences were observed between the glucose oxidase treated doughs with or without glucose, and controls. The effect of supplementation, if any, were slight. All except the loaves of the fifth treatment were scored for quality parameters by standard Crop & Food Research in-house methods. These results are shown in table 4.1.

Figure 4.3: Bread loaves baked with and without glucose and glucose oxidase.

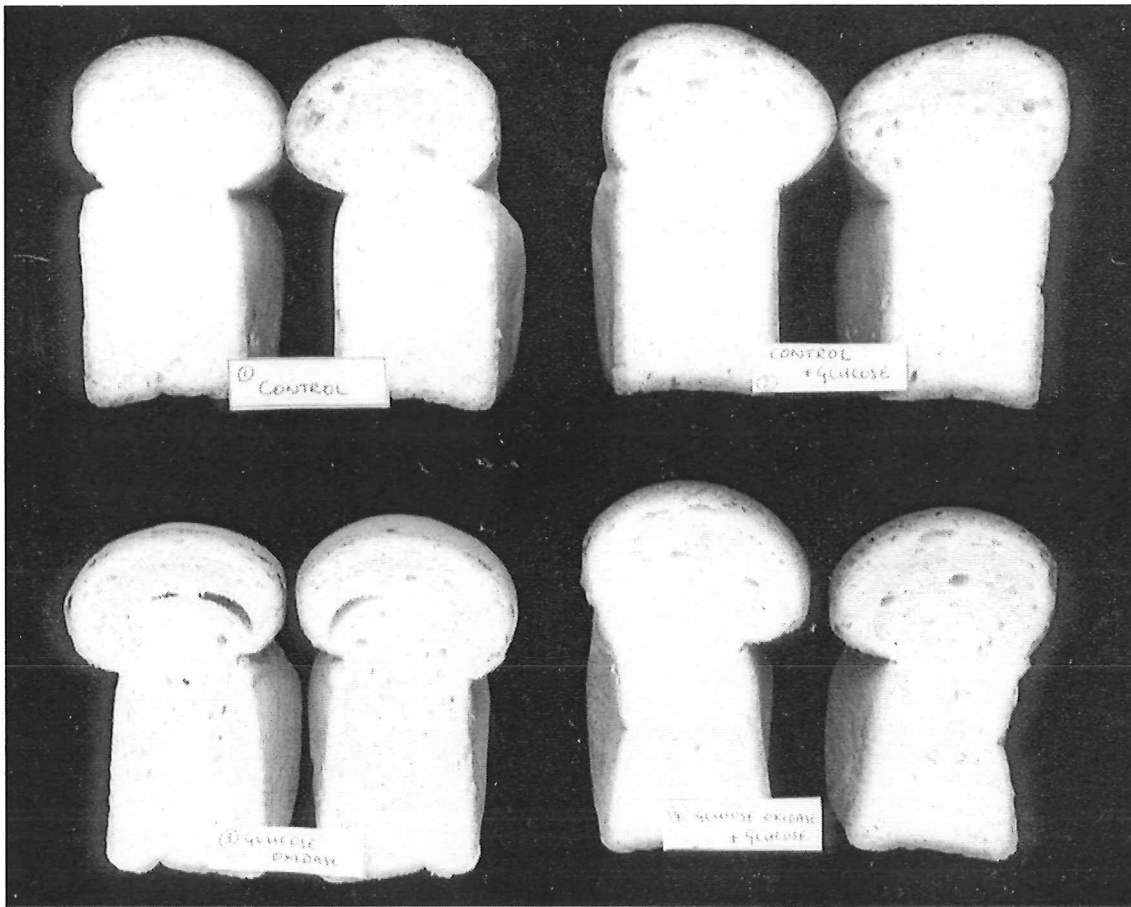


Table 4.1: Treatments and quality scores of bread loaves.

Loaf number	Description	Volume (Average of 2 measurements)	Texture (Average of 2 measurements)	Total score
1	Control	15 +/- 2	7 +/- 2	22 +/- 4
2	Control + glucose	17 +/- 2	9 +/- 2	26 +/- 4
3	Glucose oxidase	14 +/- 2	9 +/- 2	23 +/- 4
4	Glucose oxidase + glucose	14 +/- 2	9.5 +/- 2	23.5 +/- 4
5	H ₂ O ₂	-	-	-

The overall lack of change in the loaves showed that if an oxidative chemical was being produced, it did not increase loaf volume. This is consistent with literature reports. Whilst Vemulapalli and Hosney (1998) found a slight decrease in loaf volume with glucose oxidase treatment, a previous study by Kulp (1993) reported that the effect of glucose oxidase on loaf volumes was only significant when used in conjunction with a cellulase. The study of Hillhorst et al (1999) comparing doughs prepared with peroxidase, xylanase, glucose oxidase and their combinations showed that the glucose oxidase treated doughs were stronger than controls. However, the loaf volumes of glucose oxidase doughs were not significantly higher than the other doughs.

Vemulapalli et al (1998) also reported that the bread made with glucose oxidase displayed significant improvements in crumb grain. However, the glucose oxidase bread was found to be much drier than controls, which was attributed to the H₂O₂ produced by the enzyme. Whilst the best crumb texture in our work was seen in the loaves containing glucose oxidase and glucose, the fact that the increase is only barely significant indicates that the effect is minimal.

4.3.2 Effect of glucose oxidase treatment on croissant properties

The effect of TGA was most dramatically evident in the increase in volume of baked croissants. This was related to the crosslinking of the SDS-insoluble glutenin fraction. Thus it was of interest to observe the result of adding glucose oxidase to croissant formulations.

Croissants were prepared according to the standard Crop & Food in-house method. Four treatments were investigated with five croissants baked with each treatment. Treatment one being the control, treatment two had added 0.5% glucose. Treatments three and four were glucose oxidase (10 p.p.m.) and glucose oxidase (10 p.p.m.) plus added glucose (0.5%), respectively. Representative baked croissants from these treatments are shown in figures 4.4.

In sharp contrast to TGA-treated croissants, the croissants prepared with glucose oxidase showed no significant increase in volume. An overall observation of the baked croissants was a loss of shape of croissants containing glucose oxidase. This was also evident in the glucose oxidase + glucose treatment. Average values for weight and size-related parameters, calculated from five representative croissants from each treatment, are shown in table 4.2.

In terms of overall texture and crumb patterns, the best results were obtained with the control + glucose treatment. This was also the treatment that produced the most desirable "honeycomb" type pattern (figures 4.5 b). However, with the addition of glucose oxidase, some improvements from the control were observed, particularly producing a thicker, moister and more glutenous crumb as well as an opening of the size of the gas cells. In addition to this, the layers were better formed than the controls. An increased flakiness of the crust was also observed.

Figure 4.4: Croissants baked with and without added glucose and glucose oxidase.

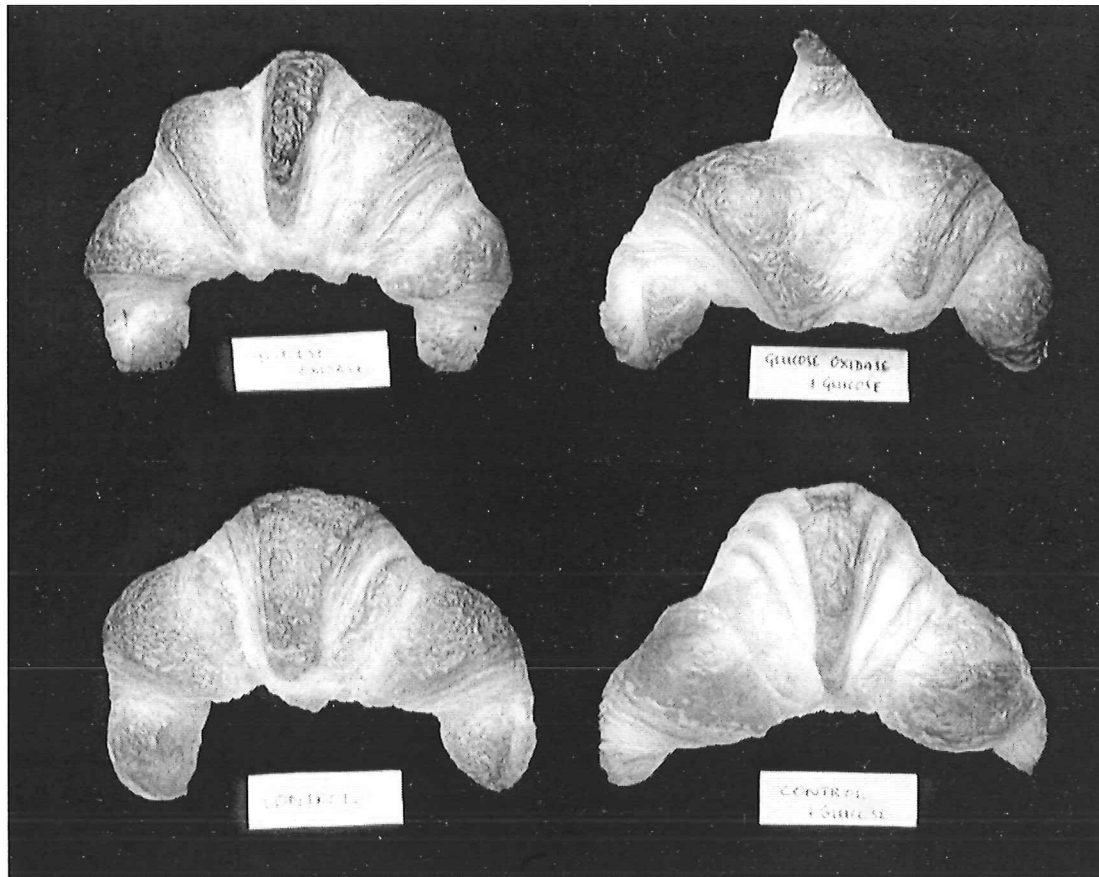


Figure 4.5 (a): Cross section through control croissants

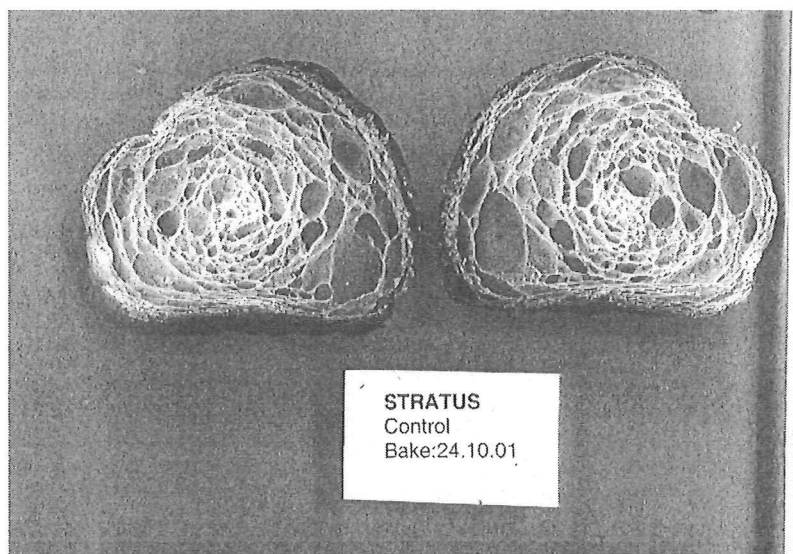


Figure 4.5 (b): Cross section through croissants prepared with extra glucose

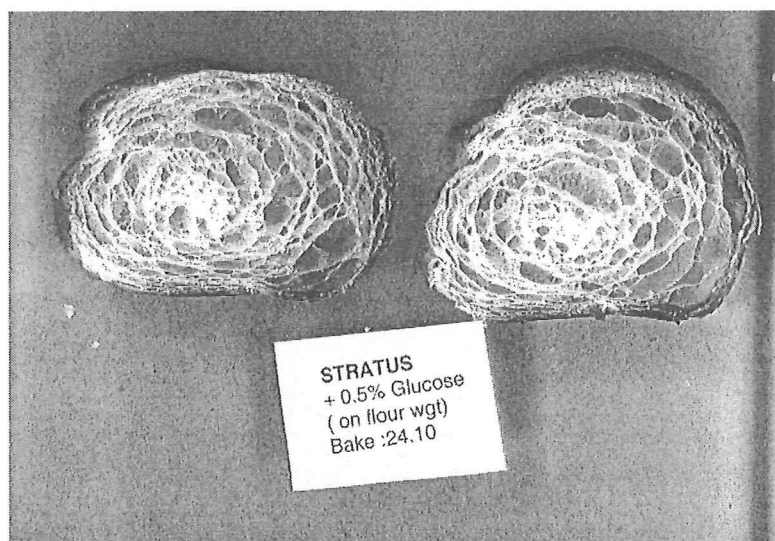


Figure 4.5 (c): Cross section through control croissants treated with glucose oxidase

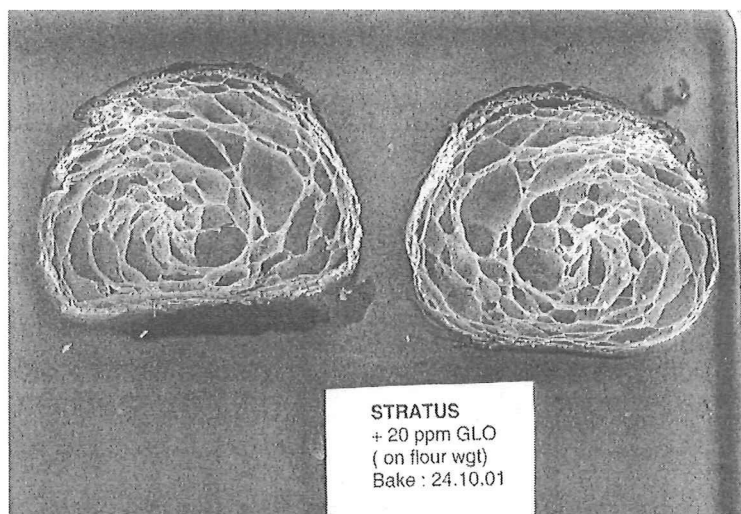


Figure 4.5 (d): Cross section through control croissants treated with glucose oxidase and glucose

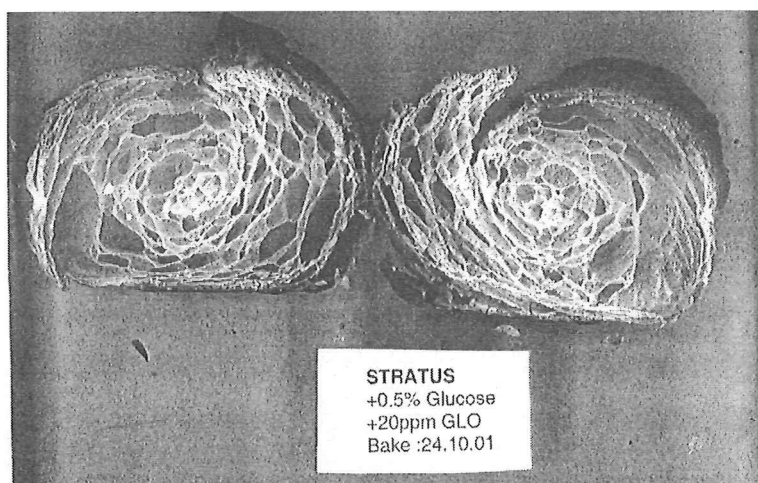


Table 4.2: Weight and volume measurements of baked croissants. Figures in brackets represent the standard error of the mean of five measurements. Volume was calculated according to the formula $\frac{2}{3} \pi W.H.L$, where W= weight, H=height and L= length (Gerrard et al, 2000).

Treatment	Fresh weight (g)		Baked weight (g)		Volume (mL)	
Control	62.1	(0.13)	50.3	(0.12)	927	(26.6)
Control + glucose	62.4	(0.15)	51.5	(0.17)	919	(26.6)
Glucose oxidase	62.4	(0.15)	50.8	(0.18)	937	(47.6)
Glucose oxidase + glucose	62.2	(0.14)	50.9	(0.25)	918	(35.8)

These effects, which were consistently observed in the croissants containing glucose oxidase alone, were softened in those which had both glucose oxidase and added glucose, which, for example, had a less flaky crust. To our knowledge, there are no previous literature reports of the improving effects of glucose oxidase on croissants.

Thus in summary, the effect of glucose oxidase was small in both bread and croissants. Product volume was unaffected, in particular contrast to the dramatic effects produced by TGA. We sought to analyse the changes caused by glucose oxidase treatment at the molecular level, by SDS-PAGE and HPLC.

4.4 Analysis of molecular changes to bread proteins following treatment with glucose oxidase

Bread dough samples from different treatments were submerged in liquid nitrogen, followed by freeze-drying and protein extraction, as for the TGA doughs described in *Chapters Two and Three*. Results from SDS-PAGE and SE-HPLC analyses of these samples are discussed below. As in our analysis of the effects of TGA on dough proteins,

samples were taken before and after proofing in order to detect any changes during this point in the processing.

4.4.1 Analysis by SDS-PAGE

Two doughs were made for each of the following treatments: (1) Control, (2) glucose oxidase non-proofed, (3) glucose oxidase proofed 23°C one hour, (4) glucose oxidase plus glucose non-proofed, (3) glucose oxidase plus glucose proofed and (5) H₂O₂. Samples were then analysed on SDS-PAGE, reduced and unreduced.

The following figures show the individual protein fractions from the different treatments, on reduced and non-reduced SDS-PAGE. Non-reduced SDS-PAGE being inherently more difficult to run than when samples are reduced, the gels of non-reduced samples were generally less well resolved than their reduced counterparts. However, the occurrence of disulfide crosslinking could be unambiguously demonstrated.

Albumins and globulins

Figures 4.6 and 4.7 show the results of the albumins and globulins run unreduced and reduced on SDS-PAGE. It is clear that the addition of glucose oxidase has resulted in the crosslinking of the albumins and globulins, as seen by the smearing and aggregated material at the top in lanes 3-6 of both gels. This result is in agreement with Vemulapalli & Hoseney (1998) who reported that glucose oxidase affected the water-soluble fraction of wheat flour. Our results suggest that the effect of glucose oxidase on the albumin/globulin fraction is significant, and that the effect appears to involve both disulfide and some non-disulfide crosslinking. This finding was not reported by Vemulapalli & Hoseney (1998).

The minimal loss of the smearing effect upon reduction of the samples, in figure 4.7 suggests that in the albumin and globulin fraction of glucose oxidase treated doughs, the formation of non-disulfide crosslinkages is more prevalent than disulfide bonding. Variables such as proofing and the addition of extra glucose appear to have had no added effect.

Figure 4.6: Non-reduced SDS-PAGE of the albumins and globulins from control, glucose oxidase and H_2O_2 treated doughs. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** glucose oxidase treated, non-prooved; **Lane 4:** glucose oxidase treated, prooved; **Lane 5:** glucose oxidase plus 0.5% glucose non-prooved; **Lane 6:** glucose oxidase plus 0.5% glucose prooved; **Lane 7:** H_2O_2 treated.

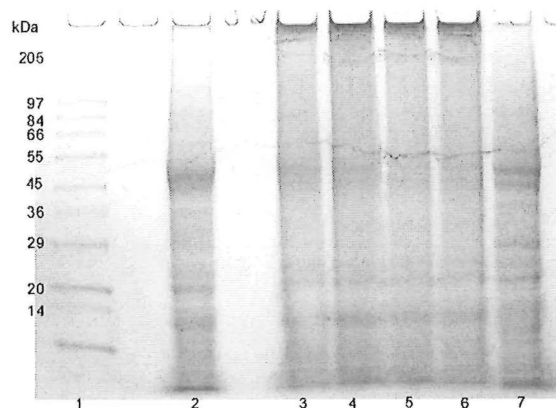
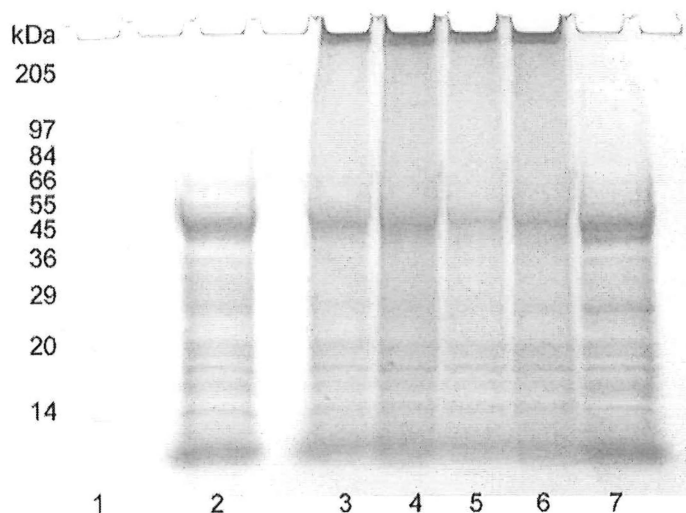


Figure 4.7: Reduced SDS-PAGE of the albumins and globulins from control, glucose oxidase and H_2O_2 treated doughs. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** glucose oxidase treated, non-prooved; **Lane 4:** glucose oxidase treated, prooved; **Lane 5:** glucose oxidase plus 0.5% glucose non-prooved; **Lane 6:** glucose oxidase plus 0.5% glucose prooved; **Lane 7:** H_2O_2 treated.



The albumin and globulin fractions of wheat flour contain higher amounts of total cysteine (6.2 and 5.4 g/16 g nitrogen) than the gluten proteins (2.7 g/16 g nitrogen for gliadin and 2.2 g/16 g nitrogen for glutenin), as reported by Bushuk and Wrigley (1974). Thus, it is reasonable to expect a greater involvement of these small proteins in oxidative crosslinking, than the other protein groups. However, the occurrence of non-disulfide crosslinking as evidenced by the reduced SDS-PAGE gels implies that the improving effect of glucose oxidase also includes other reactions, not previously reported.

In contrast to the glucose oxidase lanes in both figures, no crosslinking is evident in lane 7, representing the H₂O₂ treatment. Band intensities in this lane have remained much the same as in the control lane, despite the high dose of H₂O₂ employed. Thus, whereas H₂O₂ treatment produced dry doughs that were difficult to handle, this does not appear to have affected the albumin/globulin fraction. This is most interesting in contrast to the report of Vemlapalli and Hoseney (1998) suggesting that the production of H₂O₂ was responsible for the decrease in the thiol content of the water-soluble fraction, possibly due to their oxidation into disulfide linkages. Our findings indicate instead, that H₂O₂ production does not cause either type of crosslink to form in the albumins and globulins, at least not when added to the dough at the onset of mixing.

As for the nature of these non-disulfide linkages, it has been suggested that several types of crosslinks, may be produced by oxidation in doughs (Miller and Hoseney, 1999). The formation of crosslinks between tyrosine residues as reported by Tilley et al (2001) and the occurrence of Maillard-type crosslinks between sugars and amine groups in the proteins (Fayle and Gerrard, 2002) seem the most likely.

Gliadins

Reduced and non-reduced gel results (figures 4.8 and 4.9) showed that there was very little crosslinking of the gliadin fraction. Gliadin subunits are small, being composed of individual polypeptide chains. The thiol groups and disulfide crosslinks are internal to the monomeric structure.

Figure 4.8: Non-reduced SDS-PAGE of the gliadins from control, glucose oxidase and H_2O_2 treated doughs. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** glucose oxidase treated, non-prooved; **Lane 4:** glucose oxidase treated, prooved; **Lane 5:** glucose oxidase plus 0.5% glucose non-prooved; **Lane 6:** glucose oxidase plus 0.5% glucose prooved; **Lane 7:** H_2O_2 treated.

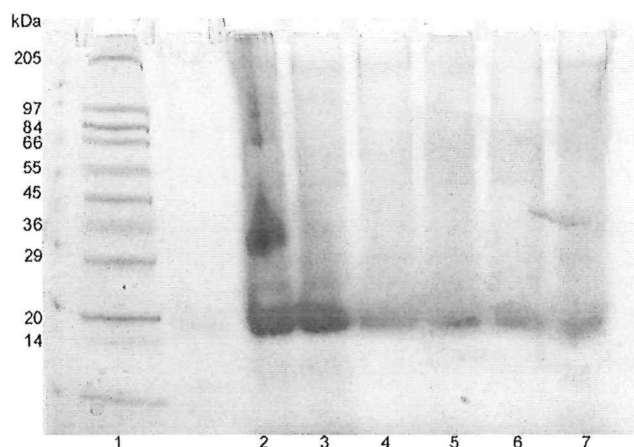
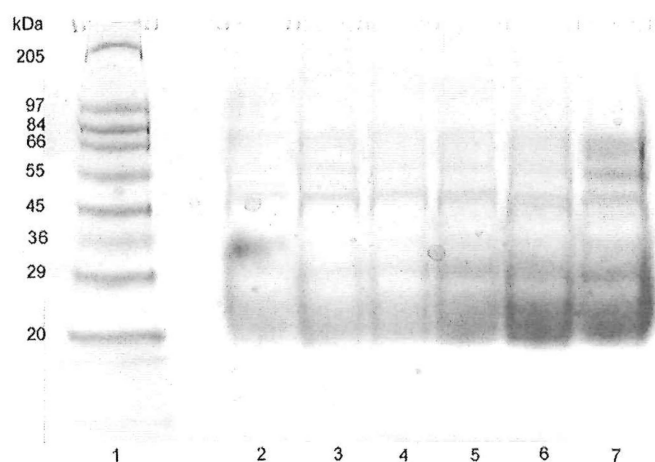


Figure 4.9: Reduced SDS-PAGE of the gliadins from control, glucose oxidase and H_2O_2 treated doughs. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** glucose oxidase treated, non-prooved; **Lane 4:** glucose oxidase treated, prooved; **Lane 5:** glucose oxidase plus 0.5% glucose non-prooved; **Lane 6:** glucose oxidase plus 0.5% glucose prooved; **Lane 7:** H_2O_2 treated.



During dough development, the compact, symmetrical structure resists stretching and linear orientation and the exposure of reactive bonding groups (Allen, 1999). For these reasons, gliadin is not readily susceptible to oxidation and the formation of disulphide crosslinks. Nor is the gliadin fraction of doughs susceptible to TGA or Maillard-type (Gerrard et al, 2002 a, b, c) crosslinking. It is thus expected that the main property of dough affected by the gliadins, namely viscous flow, would not be altered by any of these crosslinking treatments. This may be addressed in future studies.

SDS-soluble glutenins

Figures 4.10 and 4.11 illustrate the effect of glucose oxidase or H_2O_2 treatment on the SDS-soluble glutenin fraction. Non-reduced samples (figure 4.10) are heavily smeared in all lanes including the control, reflecting the polymeric nature of this fraction. Upon reduction (figure 4.11) the picture shows slightly more evidence of crosslinking in the glucose oxidase lanes than in the control. However, this is less obvious than the occurrence of non-disulfide crosslinking in the albumins and globulins. The H_2O_2 lane also has a slight indication of non-disulfide crosslinking, in contrast to the corresponding lanes in the reduced samples of the albumin/globulin and gliadin fractions.

This may be interpreted as glucose oxidase adding a small but definite amount non-disulfide crosslinks to the SDS-soluble glutenins of dough. Vemulapalli and Hoseney (1998) reported a small decrease in the solubility of the SDS-soluble group, following treatment with glucose oxidase with an accompanying reduction in the SH content (thought to be due to their oxidation into S-S bonds). We now have evidence that the effect on this fraction may also to be due to the formation of non-disulfide crosslinks.

In contrast to the lack of effect of H_2O_2 on the other fractions, it appears that H_2O_2 may add a low level of non-disulfide crosslinks to this fraction.

Figure 4.10: Non-reduced SDS-PAGE of the SDS-soluble glutenins from control, glucose oxidase and H_2O_2 treated doughs. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** glucose oxidase treated, non-prooved; **Lane 4:** glucose oxidase treated, prooved; **Lane 5:** glucose oxidase plus 0.5% glucose non-prooved; **Lane 6:** glucose oxidase plus 0.5% glucose prooved; **Lane 7:** H_2O_2 treated.

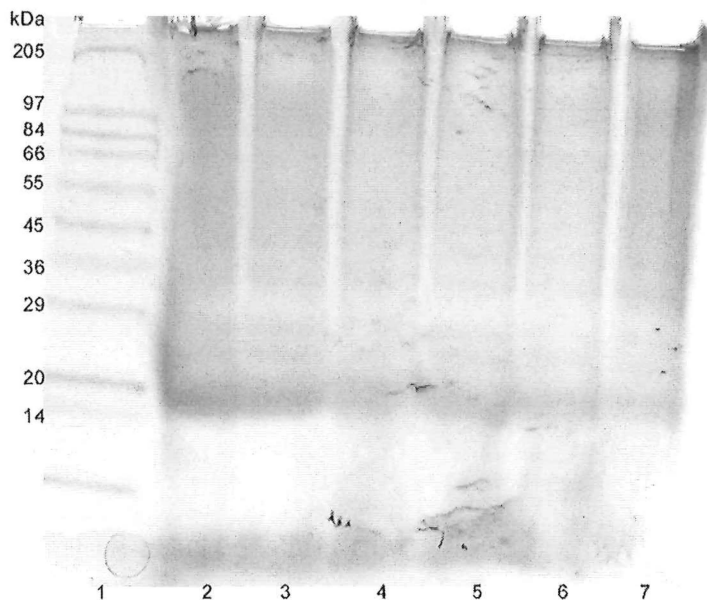


Figure 4.11: Reduced SDS-PAGE of the SDS-soluble glutenins from control, glucose oxidase and H_2O_2 treated doughs. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** glucose oxidase treated, non-prooved; **Lane 4:** glucose oxidase treated, prooved; **Lane 5:** glucose oxidase plus 0.5% glucose non-prooved; **Lane 6:** glucose oxidase plus 0.5% glucose prooved; **Lane 7:** H_2O_2 treated.

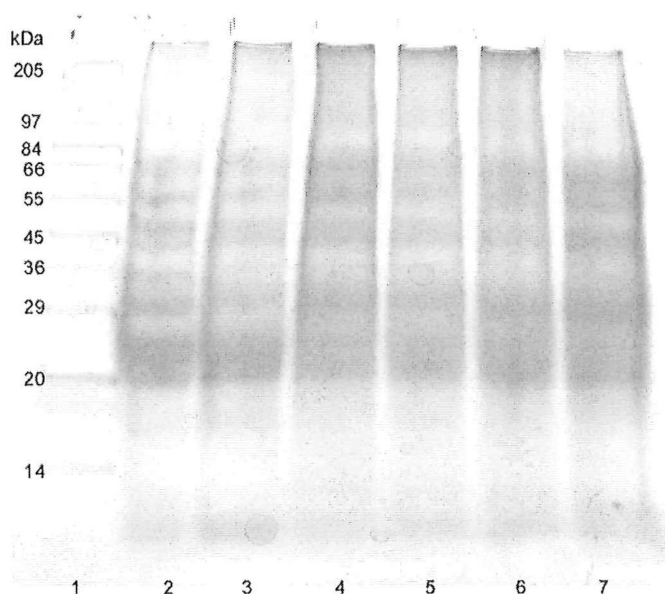


Figure 4.12: Non-reduced SDS-PAGE of the SDS-insoluble glutenins from control, glucose oxidase and H_2O_2 treated doughs. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** glucose oxidase treated, non-prooved; **Lane 4:** glucose oxidase treated, prooved; **Lane 5:** glucose oxidase plus 0.5% glucose non-prooved; **Lane 6:** glucose oxidase plus 0.5% glucose prooved; **Lane 7:** H_2O_2 treated.

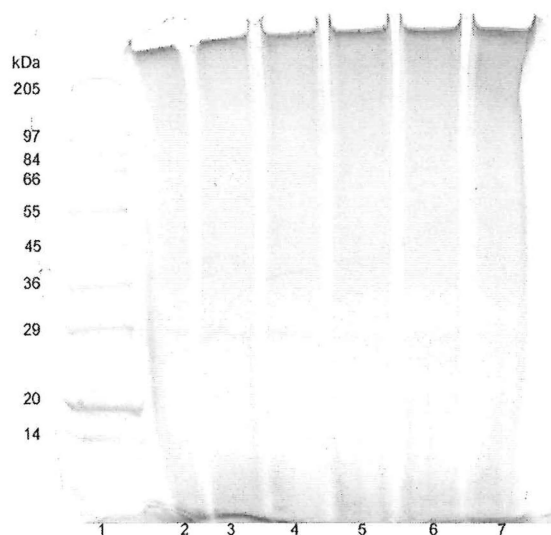
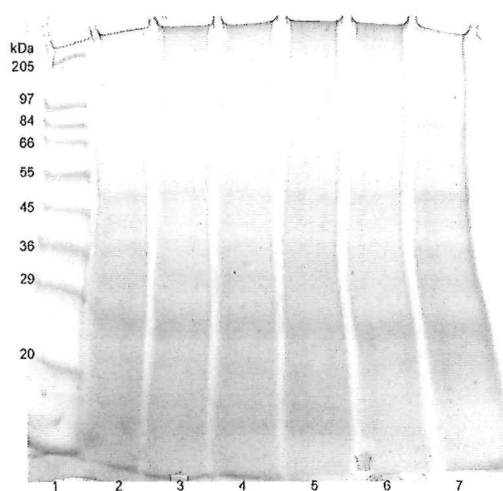


Figure 4.13: Reduced SDS-PAGE of the SDS-insoluble glutenins from control, glucose oxidase and H_2O_2 treated doughs. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** glucose oxidase treated, non-prooved; **Lane 4:** glucose oxidase treated, prooved; **Lane 5:** glucose oxidase plus 0.5% glucose non-prooved; **Lane 6:** glucose oxidase plus 0.5% glucose prooved; **Lane 7:** H_2O_2 treated.



SDS-insoluble glutenins

Figures 4.12 and 4.13 show the SDS-insoluble glutenins with and without reduction on SDS-PAGE. Whilst smearing is evident in all lanes including the control of the non-reduced gel, the reduced gel shows more evidence of non-disulfide crosslinking in the glucose oxidase lanes than in the control. As with the SDS-soluble group, this effect is less obvious than the occurrence of non-disulfide crosslinking in the albumin/globulin fraction. This leads to the novel conclusion that the effect of glucose oxidase on the SDS-insoluble glutenins includes non-disulfide crosslinking.

Vemulapalli and Hoseney (1998) found that apart from the small effect on the SDS-soluble glutenin group, glucose oxidase did not act directly on the gluten proteins, as measured by protein solubility or the relative viscosity of protein solutions. Our results show that although no extra disulfide linkages are added to the SDS-insoluble glutenin group, some non-disulfide crosslinking does take place. It is also very clear from our results, that the degree of these non-disulfide crosslinks is very much smaller than the effect of TGA on the SDS-insoluble glutenins, which is consistent with the lack of effect on quality parameters in glucose oxidase-treated products, in particular, croissant volume.

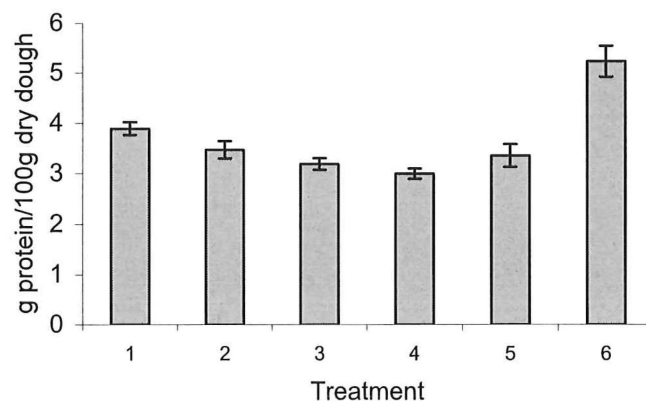
SDS-PAGE analysis: summary

The main findings from this study are, firstly that treatment with glucose oxidase appears to add both disulfide and non-disulfide crosslinks to the albumin/globulin fraction, while leaving the gliadins unaffected. Secondly, there is definite evidence of a small degree of non-disulfide crosslinking in the SDS-insoluble glutenins. Thirdly, non-disulfide crosslinks also appear to have been added to a small degree in the SDS-soluble group of gluten proteins. Finally, treatment with H₂O₂ appears to cause a slight occurrence of non-disulfide crosslinking in the SDS-soluble glutenin group.

4.4.2 Analysis by SE-HPLC

Samples from the control and treated bread doughs were analysed by SE-HPLC in order to corroborate the SDS-PAGE analysis and assess the changes in protein extractability. A stronger dough was expected to have a lower extractability of protein (Huang and Khan, 1997). Samples were analysed in triplicate and average values taken in each case. The total protein that could be extracted on SE-HPLC from each treatment is shown in figure 4.14.

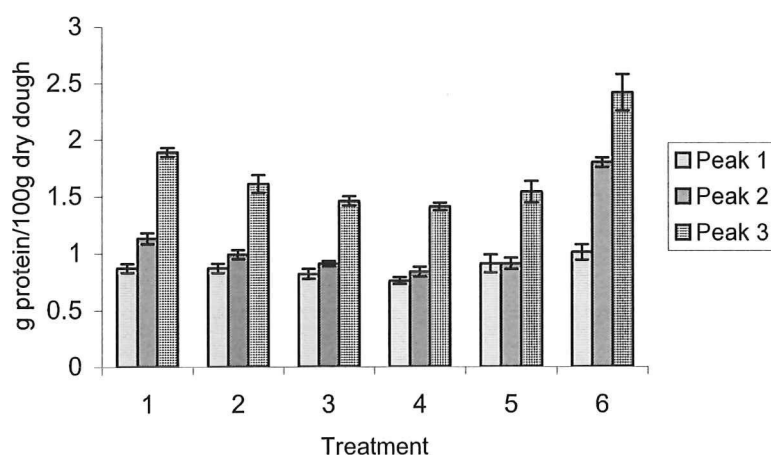
Figure 4.14: Change in the total extractable protein per 100 g dry dough for different treatments in bread doughs. Treatments: **1** = control; **2** = GO non-prooved; **3** = GO 30 minutes prooved; **4** = GO + glucose, non-prooved; **5** = GO + glucose 30 minutes prooved; **6** = H₂O₂. Error bars represent standard error of the mean of three measurements.



Treatment with glucose oxidase appeared to cause a slight reduction in the extractable protein. This was expected considering the small crosslinking effect seen on SDS-PAGE, particularly in the albumin/globulin fraction, and the corresponding minor changes in crumb texture in treated breads. The effect was most obvious in the treatment consisting of glucose oxidase and glucose (treatment 4). What could not be simply explained, was the apparent increase in the extractable protein in the H₂O₂ dough (treatment 6). As H₂O₂ was thought to strengthen the flour proteins, this dough was not expected to yield much protein on extraction. Although the reason for this result is unclear, this result was consistent with the lack of crosslinking observed on SDS-PAGE.

To ascertain the changes to specific fractions from among the different wheat protein groups, results from the various SE-HPLC peaks were analysed. As shown in figure 4.15, it was evident that the total effect was due mostly to the peak corresponding to most of the albumin and globulin fraction (peak 3), whereas large proteins (peak 1) were unaffected. No significant differences in the proteins occurred upon proving.

Figure 4.15: Changes in amount of protein in the individual peaks on SE-HPLC. Peak 1: aggregated material; Peaks 2 & 3: albumins and globulins. Treatments: **1** = control; **2** = GO non-prooved; **3** = GO 30 minutes prooved; **4** = GO + glucose, non prooved; **5** = GO + glucose 30 minutes prooved; **6** = H₂O₂. Error bars represent standard error of the mean of three measurements.



This is further evidence of the small but definite effect of glucose oxidase treatment on the albumins and globulins from dough.

SE-HPLC: Summary

SE-HPLC showed that no major change had occurred to the proteins of large molecular weight. Moreover, proving time and the addition of extra glucose substrate made no difference to the result. A small change was evident in the water-soluble fraction, particularly in the glucose oxidase + glucose treatment (treatment 4). These results corroborated the results obtained with SDS-PAGE analysis.

4.5 Crosslinking of a model protein by the glucose oxidase reaction product

In light of the demonstration of the crosslinking of albumins and globulins by glucose oxidase, it was of interest to observe the effect of this enzyme on a model albumin protein, namely ovalbumin. Ovalbumin prepared in distilled water was incubated with a preparation of glucose oxidase. Aliquots were removed at specified time intervals and the reaction stopped by the addition of treatment buffer containing 2-mercaptoethanol. The samples were also placed in ice when the reaction was halted. These samples were then analysed under reducing conditions on SDS-PAGE.

Figure 4.16 clearly demonstrates the gradual disappearance of the ovalbumin band over time and the formation of crosslinked products (A). This reaction models the crosslinking of albumins and globulins in dough containing glucose oxidase. The occurrence of the enzyme (B) between 66 and 84 kDa, is further illustrated in the figure of glucose oxidase alone (figure 4.17).

4.6 Conclusions drawn from this study

The flour-improving action of glucose oxidase has been previously ascribed to the H_2O_2 produced during the oxidation of glucose. Most of the literature reports have noted that the improvements are related to crumb texture and strength, and product volume has not been observed to increase with glucose oxidase treatment. Our study of glucose oxidase has shown that the macroscopic effects of adding this enzyme to baked products are small in comparison with those of TGA. The relatively minor effects to the dough proteins observed at the molecular level add support to this finding. However, most notable of the molecular changes are the crosslinking of the albumin/globulin fraction with both disulfide and non-disulfide bonds, and the slight occurrence of non-disulfide crosslinking in the gluten proteins, both of which are novel findings. These crosslinks are as yet unidentified, but both dityrosine and Maillard crosslinks are suggested.

Figure 4.16: SDS-PAGE of ovalbumin incubated with glucose oxidase over time. **Lane 1:** Sigmamarker; **Lane 2:** control; **Lane 3:** 5 minutes; **Lane 4:** 30 minutes; **Lane 5:** 1 hour; **Lane 6:** 2 hours; **Lane 7:** 3 hours. (O is the ovalbumin control; A represents the enzyme glucose oxidase crosslinked products; B shows the crosslinked products).

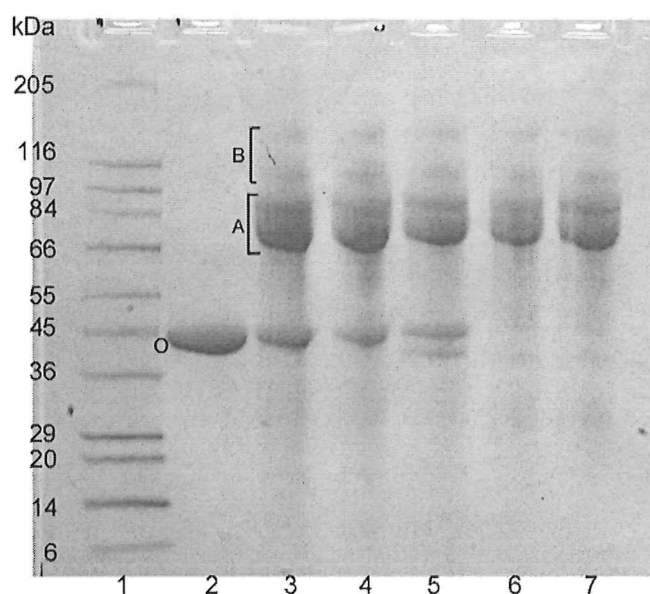
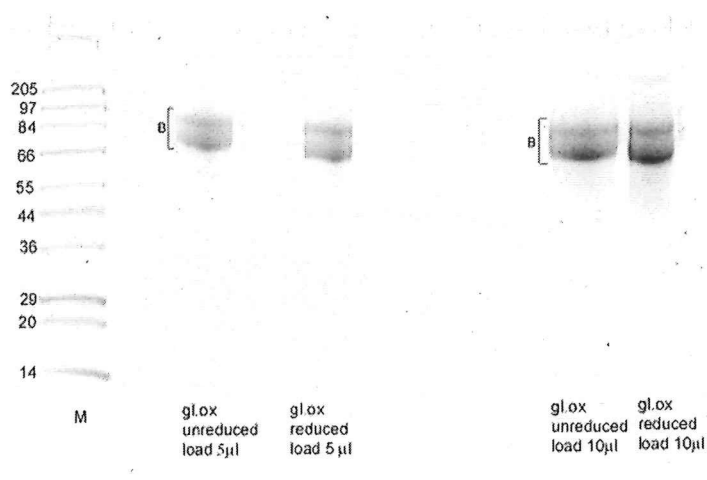


Figure 4.17: Glucose oxidase (shown in area B) run non-reduced and reduced on SDS-PAGE.



Interpretation of these glucose oxidase results, in light of our findings with TGA, as well as with other crosslinking work in our laboratory leads to some interesting conclusions. It has already been established that TGA affects mainly the SDS-insoluble glutenins and, to some extent, the albumins and globulins. Crosslinking of the SDS-insoluble glutenins into enormous complexes resulted in greatly increased dough strength in bread, and caused a dramatic increase in the volume of croissants. Other improvements, particularly relating to crumb quality, were thought to be due to the effect of TGA on the smaller, water-soluble (albumin/globulin) group.

Other studies with chemical carbonyl compounds (Gerrard et al, 2002 a, b, c) found that glutaraldehyde added to dough, crosslinked only the albumin/globulin fraction, leaving the large, gluten-forming proteins unaffected when added to doughs *in situ*. This resulted in no major changes to the dough strength of bread, or to the volume of croissants, but brought about significant improvements to crumb textural properties.

The reaction catalysed by glucose oxidase was found, in this work, to cause small improvements to crumb properties in both bread and croissants. Molecular studies established that it was mainly the albumins and globulins that were crosslinked and that this included both disulfide and non-disulfide crosslinks. A lesser occurrence of non-disulfide bonding was also identified in the glutenin proteins, which did not appear sufficient to affect croissant volume to the same level as TGA.

Answering the questions posed at the outset of this study, clearly it is crumb properties, not overall dough strength or product volume that are affected by the glucose oxidase reaction. Of the protein fractions, the most obviously crosslinked are the albumins and globulins. This has been demonstrated to involve more than one type of crosslinking. It may thus be hypothesised that it is the particular protein group becoming crosslinked, rather than the type of crosslink or the crosslinking agent employed, that is important in the improvement of specific functional properties. Obviously, different agents target different protein groups, with the consequent changes in different functional properties. The crosslinking of the water-soluble albumins and globulins, whether by TGA, glucose oxidase or chemical crosslinkers, results in particular improvements to textural properties in baked products. In contrast, a dramatic increase in the strength of the gluten network,

reflected in dough properties in bread or product volume in croissants, correlates with the crosslinking of the SDS-insoluble glutenins of wheat-based dough.

The findings from this work have thus provided a greater understanding of the mode of action of crosslinking enzymes, relating specific changes in protein structure to particular functional effects. This information should prove extremely valuable for those designing new products, or hoping to improve traditional foods, using protein crosslinking technology.

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Chapter Five

MEASUREMENT OF TRANSGLUTAMINASE ACTIVITY

5.1 Introduction

In the preceding chapters, the molecular mechanisms by which TGA and glucose oxidase brought about improvements to bread and croissants were described. The crosslinking of specific proteins was identified and related to particular changes in dough and baked products.

The different modes of action of these enzymes produced vastly different effects, both in the proteins that were crosslinked and in the resulting improvements to baked products. The direct, non-disulfide crosslinking of wheat flour proteins mediated by TGA had a much more dramatic effect than the indirect mechanism of glucose oxidase, which included both disulfide and non-disulfide crosslinking. As such, TGA proved more promising for utilization as an enzymatic flour improver. The remainder of this research was therefore centered upon further understanding the activity of TGA on food protein substrates.

When using enzymes in foods, it is important to be able to detect the presence of the enzyme and know at what level it is active in food products (Ohtsuka et al, 1996). Two considerations with TGA are firstly, to be able to detect the enzyme in commercial products and secondly, to follow the progress of the enzymatic reaction on specific food proteins, in order to better understand its mode of action.

Various assay methods for TGA have been reported in the literature. Many indirect methods, including the incorporation of amines into protein substrates (Fink et al, 1992; Wilhelm et al, 1996) have been used to quantitatively measure the activity of

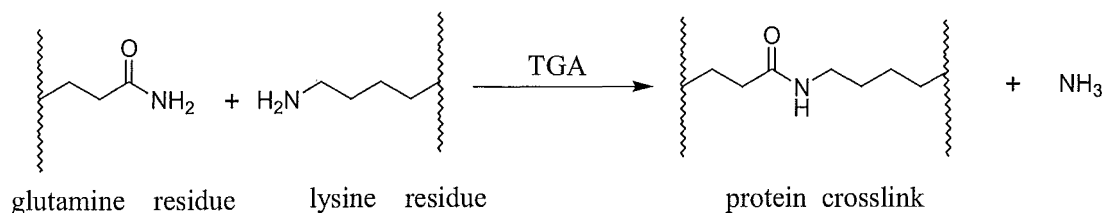
TGA. However, the incorporation of labelled amines is perhaps the least relevant of the three TGA activities (figure 1.9) in foods. It is of more interest to measure the activity of protein crosslinking or lysine loss directly. In this part of the research, both of these activities of TGA are addressed.

Wheat flour proteins are considered to be poor quality proteins primarily because of their low level of the essential amino acid lysine (Friedman and Finot, 1990). The nutritional availability of lysine in wheat products is further reduced by conditions of processing and storage, mainly due to the reaction of the ϵ -amino group of lysine with other compounds in the food or feedstuffs (Moughan and Rutherford, 1996). TGA has previously been shown to protect lysine residues within the γ -(ϵ -glutamyl)lysine crosslink and leave them nutritionally available (Seguro et al, 1996). Thus we considered that, whilst treatment with TGA would apparently reduce the level of free lysine due to their participation in crosslink formation, these residues would be accessible nutritionally. Therefore, we investigated ways by which the lysine content of individual protein fractions could be measured, as an indicator of the extent of lysine protection *via* crosslinking.

5.2 TGA activity assays

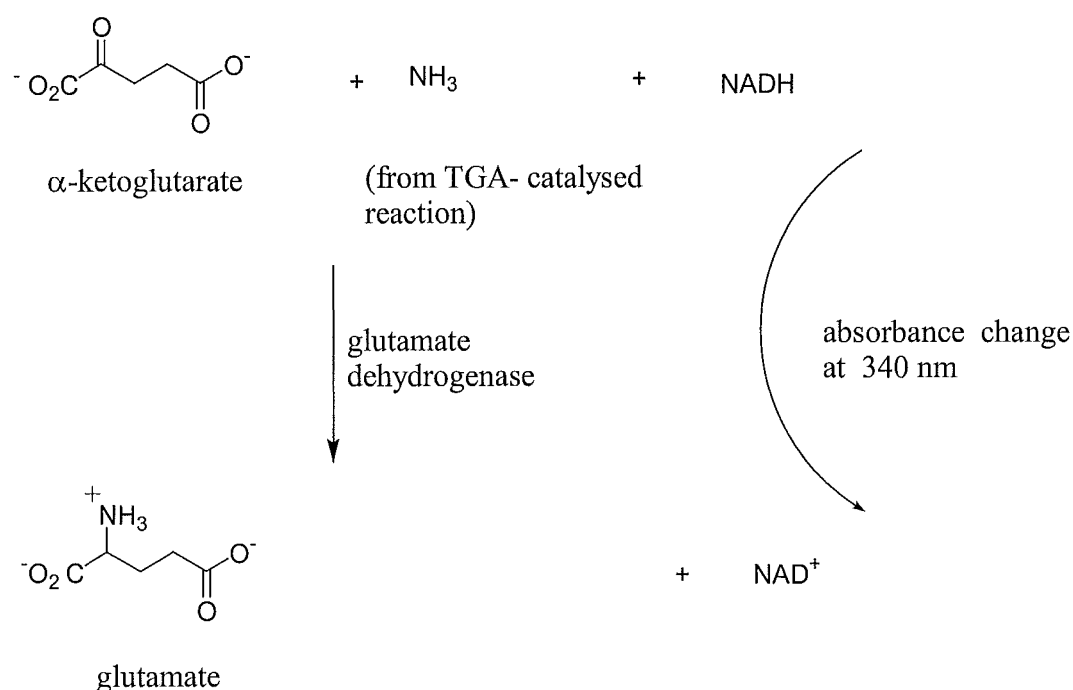
Assays reported in the literature for TGA fall into three general categories. Firstly, there are those that measure fibrin clot stabilisation and are employed in the measurement of tissue factor XIII (Jeon et al, 1989; Song et al, 1994); these are clearly inappropriate for use within food systems. Secondly, there are those assays which measure the ammonia released as a result of the crosslinking reaction (figure 5.1), as well as during amine incorporation and hydrolysis, such as that used by Day and Keillor (1999).

Figure 5.1: Release of ammonia (NH_3) during the crosslinking of glutamine and lysine residues in food, by TGA.



In order to measure the ammonia released, the above reaction is coupled to another enzyme-catalysed reaction, with glutamate dehydrogenase. This second reaction involves the conversion of α -ketoglutarate to glutamate, with the associated oxidation of the cofactor NADH to NAD^+ , as shown below (figure 5.2). As the rate-limiting step in the coupled assay is the production of ammonia, the measurement of the second reaction spectrophotometrically at 340 nm gives an indication of the crosslinking rate.

Figure 5.2: Reductive amination of α -ketoglutarate by glutamate dehydrogenase (Day and Keillor, 1999).



The above method was previously tested in our laboratory, but while it did give a measure of TGA activity, the results were found to be non-reproducible, especially in chemically ill-defined systems, such as foods.

The third category of TGA assay methods measures the incorporation of amines into a protein substrate. Amine incorporation assays are the most common way of measuring TGA activity, and may be based on colorimetric (Wilhelm et al, 1996; de Macedo et al, 2000), fluorometric (Pasternack et al, 1997), electrophoretic (Fink et al, 1992) or immunochemical (Slaughter et al, 1992; Ohtsuka et al, 1996) methods. In most of these methods, a fluorescently labelled primary amine derivative or a radio-labelled ^{14}C or ^3H is incorporated into a protein acceptor (Wilhelm et al, 1996). For example, in fluorometric assays such as that described by Fink et al (1992), a fluorescent amine, monodansylcadaverine (figure 5.3), is incorporated into the synthetic dipeptide benzyloxycarbonyl-L-glutaminyglycine (CBZ-Gln-Gly). The reaction product is separated by RP-HPLC. This method requires only very small amounts of substrates and enzyme and was reportedly well suited to kinetic studies.

Similarly, colorimetric assays (Jeon et al, 1989) incorporate amine substrates such as 5-(biotinamido)pentylamine into proteins, with the spectrophotometric measurement of the resulting conjugates.

Following Fayle (1998), the initial assay selected in our studies to detect TGA in doughs was the hydroxamate assay, which also relies on amine incorporation. This assay measured the concentration of hydroxamate, formed by the TGA-catalysed incorporation of hydroxylamine into the synthetic dipeptide CBZ-glutamyl glycine (figure 5.4).

Figure 5.3: Incorporation of monodansylcadaverine into CBZ-Gln-Gly, catalysed by TGA (Fink et al, 1992)

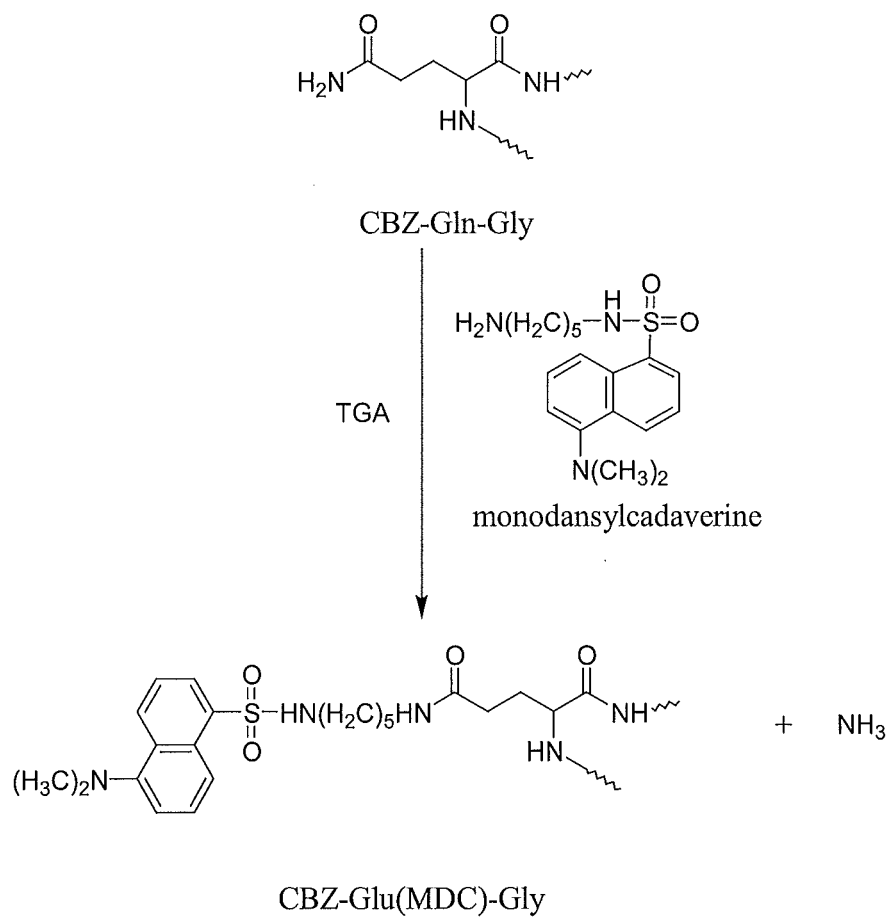
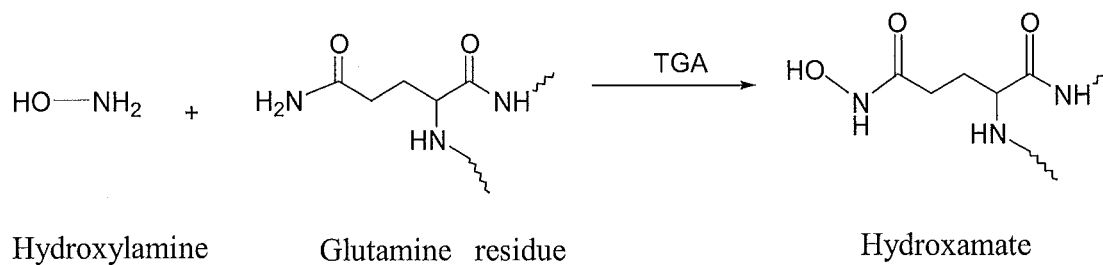
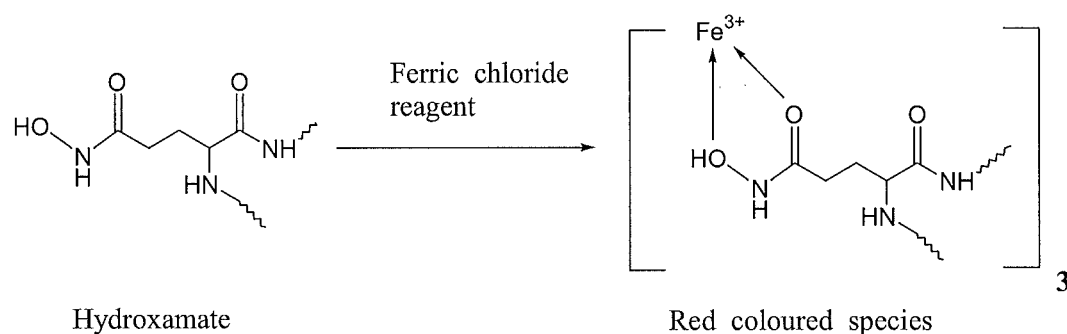


Figure 5.4: The formation of hydroxamate, catalysed by TGA.



A ferric chloride solution was added to the hydroxamate, which chelated hydroxamate but not hydroxylamine (figure 5.5), forming a red precipitate. This product was measured colorimetrically by a change in absorbance at 525 nm.

Figure 5.5: The formation of a chelated complex by ferric chloride.



We initially tested this assay with varying concentrations of TGA in distilled water. A standard curve was established, as shown in figure 5.6. This was followed by measurement of TGA in the samples of bread or croissant dough. To do this, an aqueous extract was prepared from the TGA-treated dough being measured. This was added to the cuvette containing the reaction mixture. Following incubation, the ferric chloride solution was added and the colour change measured at 525 nm.

Figure 5.6: Standard curve for TGA. Error bars represent standard error of the mean of duplicate readings.

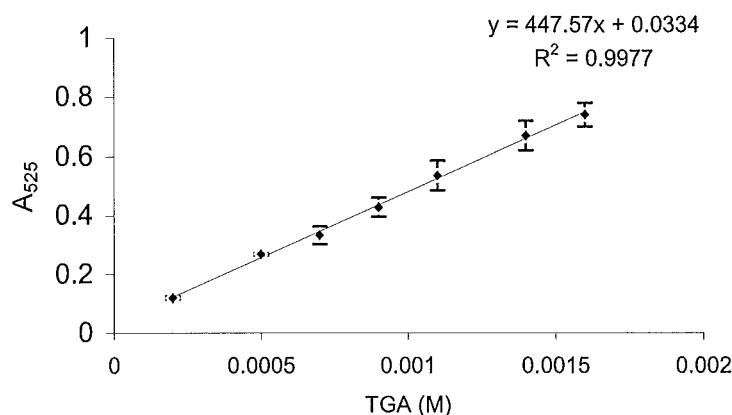
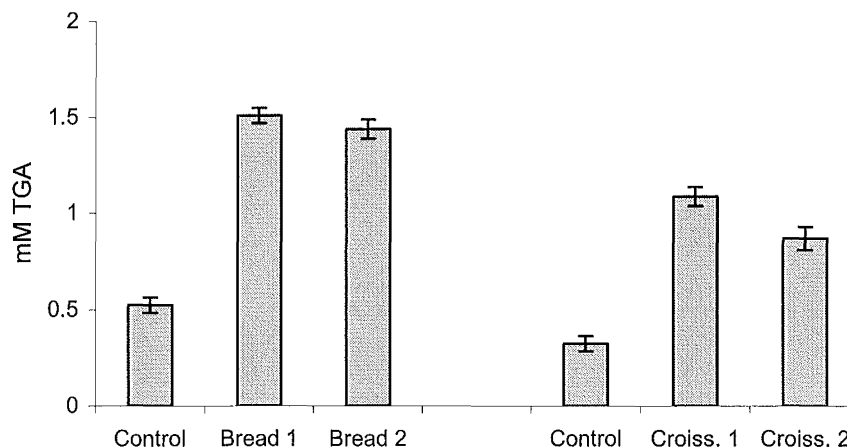


Figure 5.7: TGA concentrations measured with the hydroxamate assay, in two separate doughs of bread and croissants, all treated with 5000 p.p.m TGA, proofed and frozen for 8 months. Error bars represent the standard error of the mean of duplicate readings.



The hydroxamate assay clearly demonstrated the successful extraction of active TGA from both bread and croissants, at levels above the background levels shown in the control (figure 5.7), which were attributed to interfering substances extracted from the flour. The levels from croissant doughs were significantly lower than bread, presumably reflecting a lower extractability and / or denaturation of the enzyme in the presence of high fat concentration. That the enzyme remains active in the dough is interesting, in view of the fact that its action seems complete before proofing. This suggests that the reaction stops due to lack of available substrate, rather than loss of enzyme activity.

Since the presence of TGA was measured successfully, it was concluded that the hydroxamate assay could be employed for the measurement of TGA in commercial doughs. This was the initial requirement in our study of TGA activity. The assay was quick, simple and readily adapted for the measurement of the enzyme in bakery products. However, although a semi-quantitative measure of TGA was obtained with this method, it was not a direct indicator of the crosslinking activity of this enzyme on the constituent proteins. For detailed mechanistic studies, a more suitable method was required for measuring the crosslinking activity on a specific substrate. Prior to the development of such a method, we sought to examine how the presence of TGA in a

dough affects the concentration of the amino acid lysine, a participant in the crosslinking reaction.

5.3 Measurement of the effect of TGA on the lysine residues in dough proteins

5.3.1 Measuring lysine in food protein

Chemical estimation of lysine in foods can be achieved by the measurement of total lysine or reactive lysine. Total lysine is generally determined after acid hydrolysis treatment, but it does not necessarily reflect the amount of nutritionally available lysine (Albala-Hurtado et al, 1997). A number of chemical methods have been developed for the estimation of reactive lysine, which are those lysine residues which still possess a free unreacted ϵ -amino group (Cayot and Tainturier, 1997; Albala-Hurtado et al, 1997; Kwok et al, 1998). Such methods are presumed to give a more accurate indication of the nutritionally available lysine (Albala-Hurtado et al, 1997). Most of these methods depend on the reaction of the free ϵ -amino group of peptide-bound lysine residues with a chemical reagent (Wallace and Fox, 1998). The modified protein may then be subjected to acid hydrolysis, prior to spectrophotometric determination of the conjugated lysine.

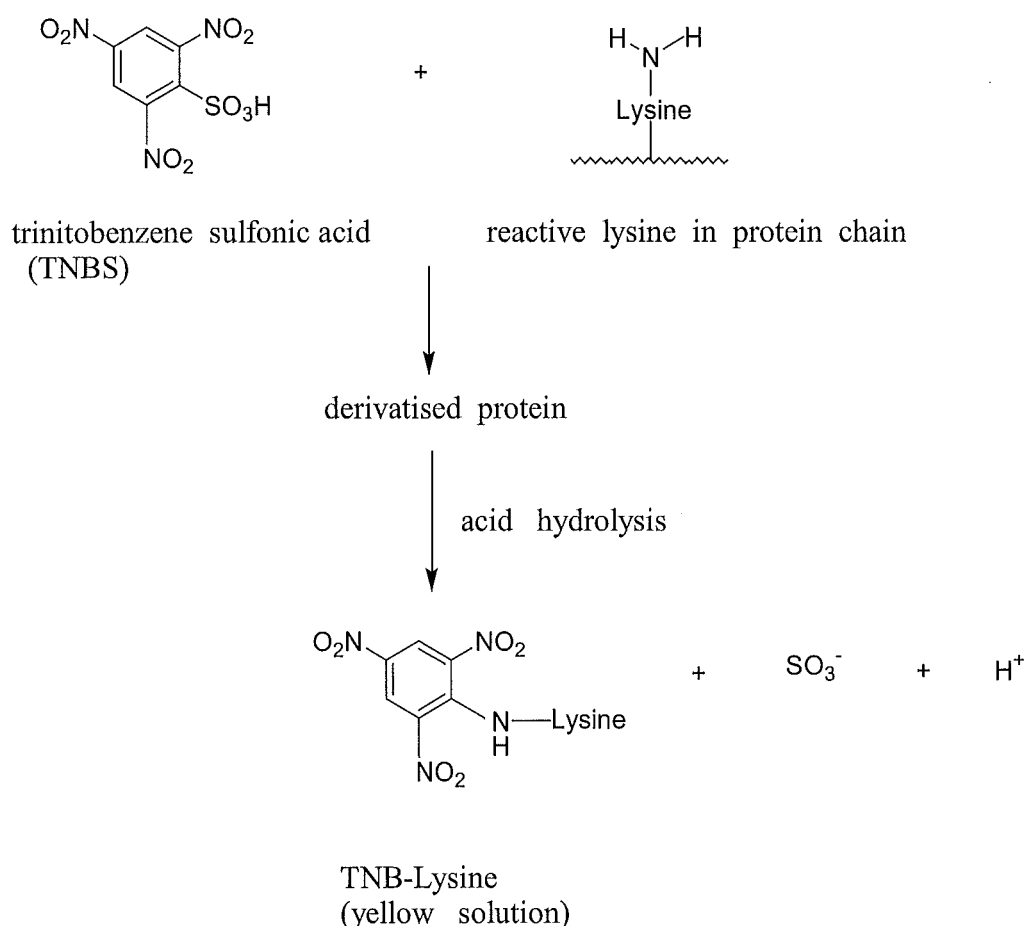
One commonly used procedure for lysine estimation in foods is the ninhydrin method (Wallace and Fox, 1998). Ninhydrin reacts with free amino groups to form a purple chromophore, which is measured at 570 nm. This method has been found to be satisfactory for use with small proteins such as ribonuclease and some dairy proteins (Fox and McSweeney 1996) and does not rely on acid hydrolysis. However, it is not as sensitive as many other chemical assays (Roth, 1971).

Another common method in use is the trinitrobenzene sulfonic acid (TNBS) method. In an adaptation of the original method developed by Carpenter (1960), trinitrobenzene sulfonic acid (TNBS) was employed to estimate reactive lysine (Hurrell and Carpenter, 1981). TNBS has the advantage over previously used reagents, such as 1-fluoro-2,4-dinitrobenzene (FNDP), in that it is water-soluble. Additionally, it is only necessary to acid-hydrolyse the TNBS-reacted material long enough to break the protein down to

soluble peptides, rather than amino acids, before the TNBS-lysine product can be measured colorimetrically (figure 5.8).

As with the original method, one difficulty with the TNBS method and other similar methods, is the interference produced from other amino acids and carbohydrates in foods (Adler-Nissen, 1979; Albala-Hurtado et al, 1997; Hurrell and Carpenter, 1981). However, they are widely used to determine free amino groups in proteins and protein hydrolysates (Adler-Nissen, 1979).

Figure 5.8: Reaction of TNBS with reactive lysine in food proteins. (Hurrell and Carpenter, 1981).

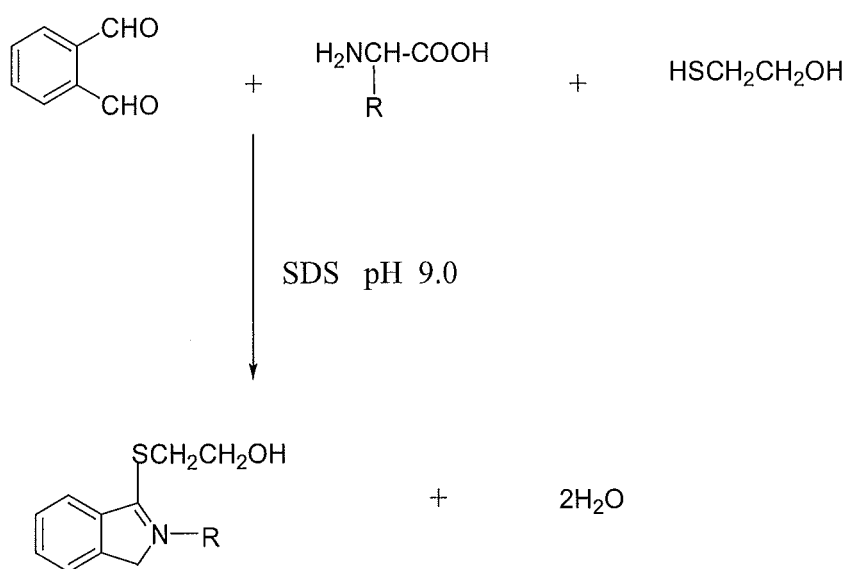


The fluorometric *o*-phthaldialdehyde (OPA) method of Roth (1971) is another method suitable for lysine measurements. The OPA method is considered by some to be advantageous over other methods due to its rapidity, simplicity and the addition of a

single prepared reagent to the sample (Goodno et al, 1981), its applicability to proteins over a broad range of lysine content, and because it does not require acid hydrolysis of the samples, minimising aretefact formation.

In an alkaline medium such as bicine (pH 9.5) containing the reducing agent 2-mercaptoethanol, OPA reacts with the free amino groups of lysine as well as peptides and proteins containing lysine (figure 5.9) (Wallace and Fox, 1998). The absorbance is measured at 340 nm. However, while it has some advantages over the methods requiring acid hydrolysis, sources of error include the necessity for precise timing and small sample volumes.

Figure 5.9: The OPA reaction. The reactive amine and the thiol compound used in the reaction are shown to react with the OPA reagent at pH 9. (Wallace & Fox, 1998).



Previously, work in our laboratory had adapted the OPA method of Bertrand-Harb et al (1993) to cereal proteins, using 50% 1-propanol as the solvent (Reid, 1999, *unpublished results*). We therefore used this as our routine method to measure lysine in the dough extracts of bread and croissants. Individual Osborne fraction extracts from frozen croissants were also analysed for their lysine content by the TNBS method, in order to corroborate the OPA results. Both methods were simple to carry out and yielded comparable results.

5.3.2 Measurement of lysine in combined protein extracts from bread dough

As well as measuring the lysine content of individual Osborne fractions, an estimation of the combined lysine in the doughs was made, in order to compare the results with those reported in the literature for wheat flour. Three control bread doughs from the same flour were each subjected to three extractions of their combined protein with a 50% 1-propanol solution, reported to be useful for extracting much of the protein from flour (Reid, 1999 *unpublished data*, Marchylo et al 1988). Lysine measurements of these extracts were taken using the OPA method and expressed as mg lysine / 100 mg protein. An overall average value of 4.82 mg lysine /100 mg was found, as shown in table 5.1.

Table 5.1: Lysine measurements of 1-propanol extracts from three doughs prepared from the same flour. (*sem = standard error of the mean of three measurements*).

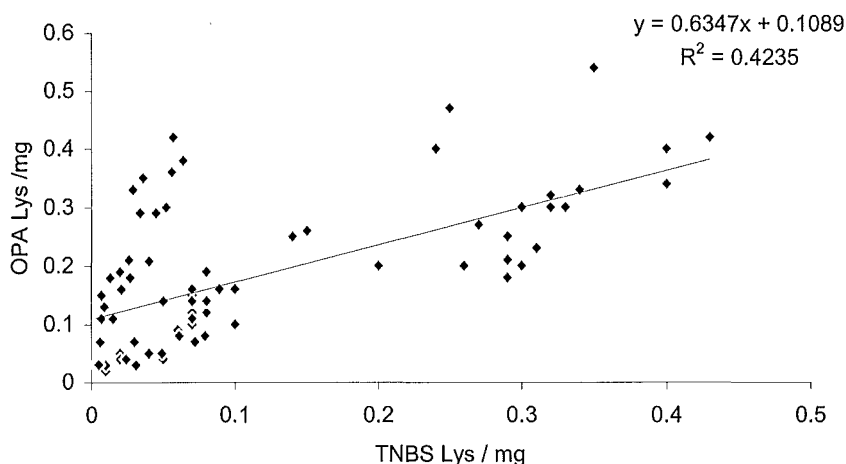
Dough	lysine mean value (mg lysine/ 100 mg)
1	4.13 (sem 0.36)
2	4.8 (sem 0.78)
3	5.3 (sem 0.41)
Average for flour	4.82 (sem 0.49)

These levels compared well with those of a recent publication (Singh et al, 2000) reporting the lysine contents of several *Triticum* wheat varieties. These researchers estimated lysine using the trinitrobenzene sulfonic acid method (TNBS), discussed above. The reported range, expressed as mg lysine/ 100 mg protein was between 2.74 and 3.64 in specially grown varieties, and between 3.36 and 6.5 for those obtained from an Australian cereals collection.

5.3.3 Comparison of lysine content of frozen croissant fractions, with the OPA and TNBS methods

Several extractions of the protein fractions were made, from the frozen croissants. The extracts of the albumin/globulins, gliadins, SDS-soluble and insoluble glutenins were analysed for their lysine concentrations by the two methods mentioned above. The purpose was to corroborate the OPA results with the TNBS method and to increase confidence in the use of both methods for dough proteins. Several runs were carried out with each method, each run being preceded by the establishment of OPA and TNBS standard curves. Protein concentrations were determined by the Bradford (1976) method. Figure 5.10 shows a comparison of results obtained by the two methods.

Figure 5.10: TNBS-OPA correlation curve for the lysine measurements of dough proteins with data from all fractions pooled.



Whilst the combined data correlating the lysine measurements by the two methods is, at first glance, disappointing, careful inspection of the individual fractions reveals some interesting trends. It becomes apparent that within each individual fraction, the correlation is much more satisfactory, as shown in the following graphs.

Figure 5.11: TNBS correlation curve for the lysine concentrations of the albumin/globulin fraction from frozen croissants.

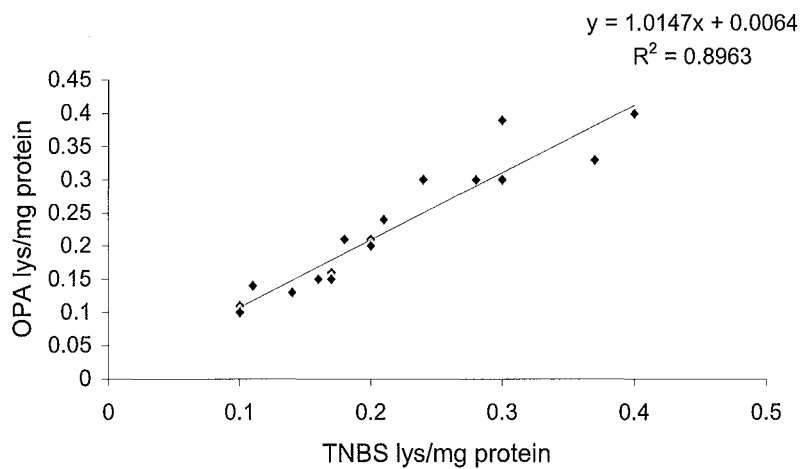


Figure 5.12: TNBS-OPA correlation curve for the lysine concentrations of the gliadin fraction from frozen croissants.

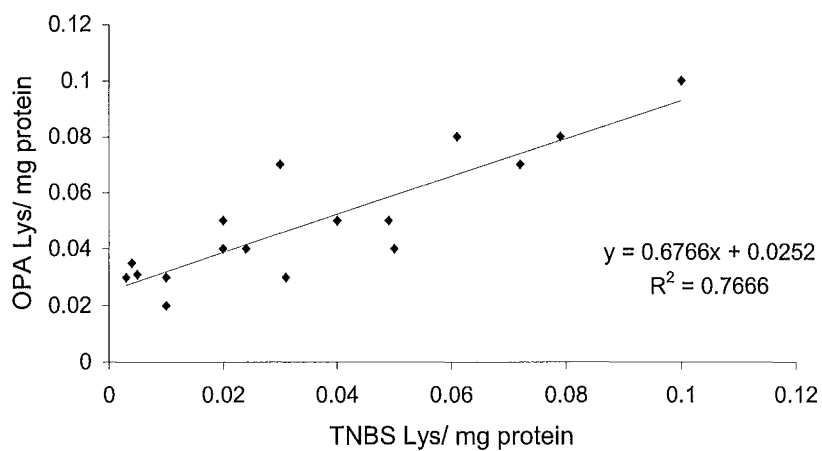


Figure 5.13: TNBS-OPA correlation curve for the lysine concentrations of the SDS-soluble glutenin fraction from frozen croissants.

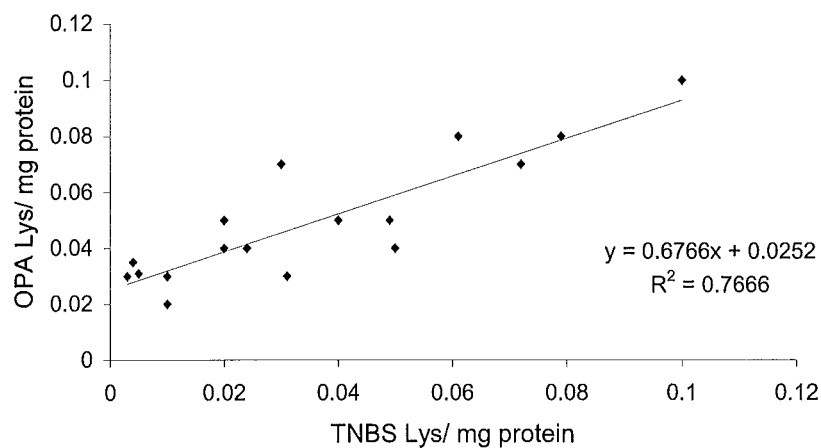
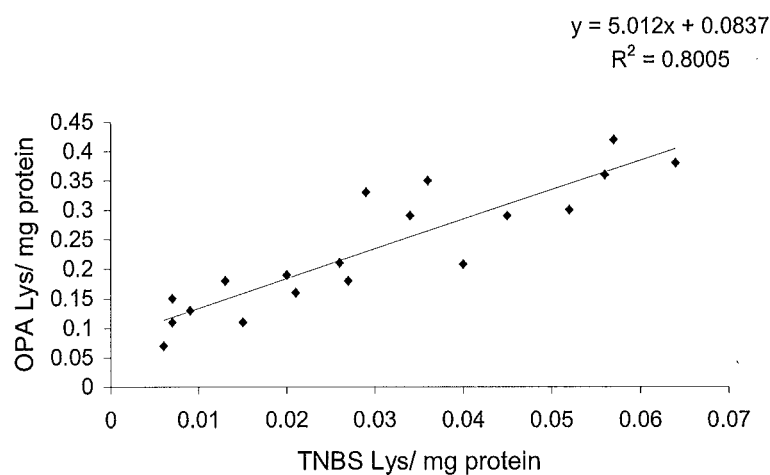


Figure 5.14: TNBS-OPA correlation curve for the lysine concentrations of the SDS-insoluble glutenin fraction from frozen croissants.



The combined data is skewed by the SDS-insoluble glutenin fraction, for which the two methods show a very poor correlation. The slope of 5.01 seen for the SDS-insoluble glutenin fraction (figure 5.14) suggests that the OPA method may be overestimating the lysine measurements while the TNBS method is possibly under-

estimating them. Such discrepancies may be expected due to the poor solubility of these large proteins.

In view of R^2 correlation levels of 0.7-0.89 between the OPA and TNBS methods for the other fractions, it was concluded that in terms of lysine measurements of dough extracts, the results obtained with the OPA method were acceptable so long as individual fractions were measured. However, it was noted that factors influencing the variability in measurements did occur, as discussed in the next section. As such, measurements of lysine levels were considered unreliable as a quantitative indicator of the crosslinking activity of TGA for mechanistic study.

5.3.4 Effect of TGA treatment on lysine content in dough

Determination of lysine concentrations in the individual Osborne fractions from control and TGA doughs was generally difficult, due to solubility problems and the possible interference caused by the different extraction solvents with the OPA solution. However, overall, the combined analysis over several hundred samples confirmed that there was a reduction in lysine due to TGA treatment, of between 10 and 20%. This reduction, which almost occurred instantaneously upon mixing, was consistent with our previous observations of the immediate action of TGA on the dough proteins as observed by SDS-PAGE and HPLC, confirming that neither proofing time nor freezing was not a determining factor. Figures 5.15 and 5.16 show that the drop in lysine has remained constant over time in both bread and croissants respectively.

Figure 5.15: % Lysine remaining compared to control doughs after TGA treatment in bread protein extracts 30 minutes of proofing at 23°C. Error bars represent the standard error about the mean of five readings.

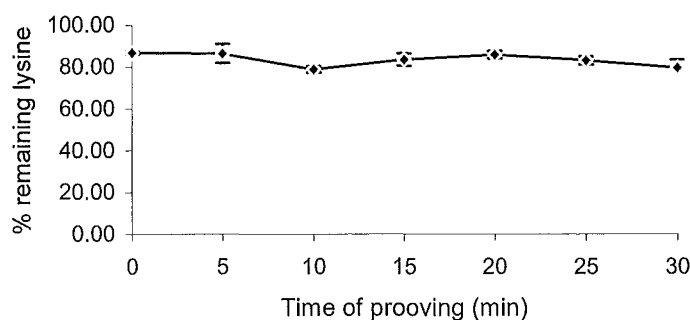


Figure 5.16: % Lysine remaining compared to control doughs after TGA treatment in croissant protein extracts over 90 days in frozen storage. Error bars represent the standard error about the mean of six readings.

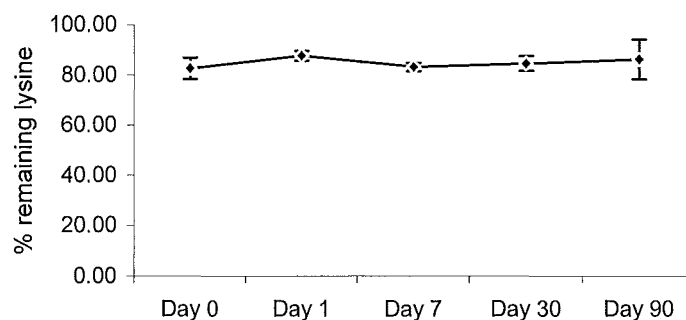


Table 5.2 confirms that an approximately 15% mean drop in lysine concentration is consistently seen on treatment with TGA.

Table 5.2: Analyses of three different bread and two different croissant preparations for the % drop in lysine following TGA treatment. OPA method used for lysine measurements of the control and TGA treated samples.

Dough	Mean % drop in lysine	No. of samples (n)
Bread set 1	13.30 (+/- 3.17)	25
Bread set 2	13.36 (+/- 3.82)	21
Bread set 3	17.70 (+/- 3.37)	13
Croissant set 1	13.30 (+/- 3.89)	28
Croissant set 2	14.55 (+/- 3.0)	28

Thus, the drop in lysine due to TGA treatment may indicate there is a certain nutritional value in the crosslinking of the dough proteins in that approximately 15% of wheat lysine residues are protected from damage during processing in a nutritionally available form. This is promising in light of the reported attempts to enhance the nutritional value of bread with added lysine (Friedman and Finot, 1990).

While the change to lysine levels indicated activity by TGA, this was of limited value in following the progress of the crosslinking reaction. Instead, we sought to develop a new novel method for measuring the crosslinking activity of TGA on the proteins of wheat dough. These studies are described in the next section.

5.4 Measurement of the rate of TGA-mediated crosslinking with model protein substrates

5.4.1 Background

Since the changes in functional properties of food proteins by TGA appear to be a result of crosslinking activity, rather than amine incorporation or lysine loss, we decided to develop a suitable assay with which the rate of the crosslinking reaction could be measured specifically. Thus in the interest of examining the substrate specificity of TGA, particularly with reference to the glutenins, we set about developing a method for investigating the rate of the crosslinking reaction with model protein substrates.

Several studies have addressed the substrate specificity of TGA (Coussons et al, 1992; Grootjans et al, 1995; Taguchi et al, 2000; de Jong et al, 2001). It has been found that the number of proteins acting as glutaminy l substrates for TGA appears to be restricted, whereas TGAses are much less selective towards the amine donor lysine residues in proteins (Grootjans et al, 1995). As well as the requirements of proteins for acting as substrates, the active site of the enzyme has been studied, both in mammalian (Pedersen et al, 1994; Weiss et al, 1998) and microbial (Kobayashi et al, 1998; Makarova et al, 1999) TGA. Our results were therefore interpreted in the context of such studies.

5.4.2 Choice of methods for crosslinking studies

Since the characteristic fading of bands on SDS-PAGE gels indicated the reduction in protein concentration due to crosslinking (Motoki and Nio, 1983; Aboumahmoud and Savello, 1990; de Jong et al, 2001), we considered that a quantitative analysis of the bands of TGA-reacted protein substrates over various time intervals would provide an

accurate indication of crosslinking rates. The suitability of this approach for the glutenins was further confirmed by the reported use of densitometry by Huang and Khan (1997) as a quantification method for wheat glutenin subunits. We considered SDS-PAGE to be more practical for the development of new methodology than HPLC, as our SDS-PAGE analyses had proved more robust and reproducible than those by RP-HPLC. SE-HPLC does not separate individual proteins and was thus unsuitable.

5.4.3 Selection of model proteins

Prior to studying the crosslinking rate of a glutenin subunit, experimental procedures were established using model proteins. For method development purposes, we chose well characterised, stable and easily identifiable proteins for SDS-PAGE analysis: ribonuclease A (RNase A), bovine serum albumin (BSA) and κ -casein.

RNase A was previously used as a model protein by Fayle et al (2000) in their studies of the crosslinking of proteins by dehydroascorbic acid (DHA). Their selection of RNase A was based on its small size (13.68 kDa), its thermal and chemical stability and the reproducibility of the results obtained. We therefore considered RNase to be a suitable protein to study crosslinking by TGA. RNase contains 11 free amino groups, from the ϵ -amino group of the 10 lysine residues plus the single terminal α -amino group (Fayle et al, 2000). Due to the hydrophilic nature of the amino group, each of the lysine residues is located on the surface of the folded protein (Boque et al, 1994). There are 7 glutamine residues in each molecule of RNase A (Garrett and Grisham, 1999).

κ -Casein was selected as a model protein due to its small size (19.02 kDa) and high contents of 12 glutamine and 9 lysine residues (Wong et al, 1996). The crosslinking by TGA of several milk proteins, including the caseins other than κ -casein, has been previously reported (Christensen et al, 1996; Haertle and Chobert, 1999; Mizuno et al, 1999; Kuraishi et al, 2001).

In sharp contrast to κ -casein, bovine serum albumin (BSA) is known to be a poor substrate for TGA (Ikura et al, 1984; Han and Damodaran, 1996). With a molecular weight of 66.43 kDa, this was the largest of the three model proteins in these studies.

5.4.4 TGA-catalysed crosslinking of model proteins

The reaction between each model protein and TGA was carried out under identical conditions of temperature and pH. The experimental procedure used is described in *Chapter Seven*. Following the reaction, samples from each set of incubations were run reduced, on SDS-PAGE.

The intensities of the bands on the SDS-PAGE gels represented the amount of protein remaining in the incubation mixture following crosslinking by TGA. Typically, a decrease in intensity was seen upon addition of the enzyme, which decreased over time at a rate which depended on the reactivity of the protein. Also evident was the accompaniment of this effect by the formation of polymeric crosslinked material at the top of the resolving gel.

Gels analysing each incubation were photographed and image analysis was performed using the Bio-Rad Quantity One Image Analysis Software Package, allowing numerical quantification of the concentration of protein in each band. Background interference was automatically subtracted from the analysis on each gel. Three incubations were carried out for each protein and TGA, followed by two gels and three analyses of each gel, as described in *Chapter Seven*.

Log values and averages were calculated and graphed against time for each incubation. Margins of error were calculated for each set of three measurements. The logarithmic slope for each set of data was taken to be representative of the reaction rate in each case.

Figures 5.17 - 5.19 show the drop in protein concentration upon incubation with TGA for the model proteins. On each gel, the control protein band is labelled A. The label B shows the last remaining protein before complete disappearance due to crosslinking. However, for RNase A and BSA (figures 5.17 and figure 5.18), the band B did not disappear completely, reflecting the lesser response to TGA of these proteins than κ -casein.

Figure 5.17: SDS-PAGE of RNase A with TGA, over time. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** incubation time 0 minutes; **Lane 4:** incubation time 30 minutes; **Lane 5:** incubation time 60 minutes; **Lane 6:** incubation time 1.5 hours; **Lane 7:** incubation time 3.0 hours; **Lane 8:** incubation time 4.0 hours; **Lane 9:** incubation time 4.0 hours.

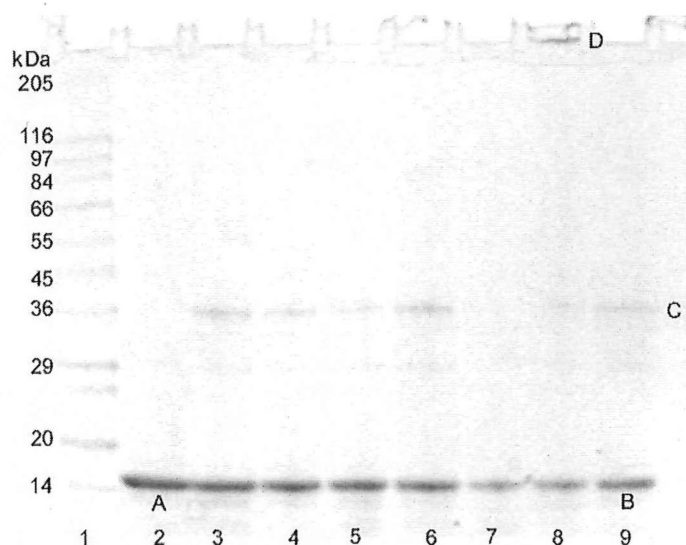


Figure 5.18: SDS-PAGE of BSA with TGA, over time. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** incubation time 0 minutes; **Lane 4:** incubation time 15 minutes; **Lane 5:** incubation time 30 minutes; **Lane 6:** incubation time 45 minutes; **Lane 7:** incubation time 60 minutes; **Lane 8:** incubation time 2.0 hours; **Lane 9:** incubation time 3.0 hours. **Lane 10:** 4.0 hours.

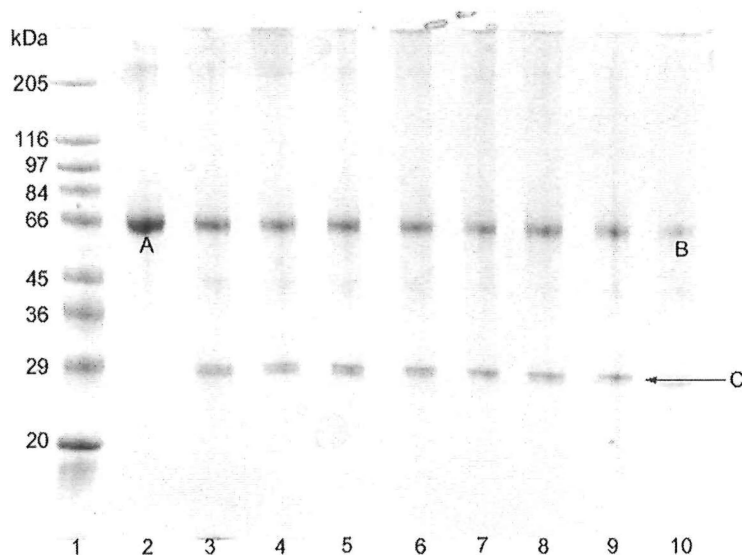


Figure 5.19 (a): SDS-PAGE of κ -casein with TGA [0.01 g / mL], over time. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** incubation time 0 minutes; **Lane 4:** incubation time 5 minutes; **Lane 5:** incubation time 15 minutes; **Lane 6:** incubation time 30 minutes; **Lane 7:** incubation time 45 minutes; **Lane 8:** incubation time 60 minutes; **Lane 9:** incubation time 1.25 hours. **Lane 10:** 1.5 hours.

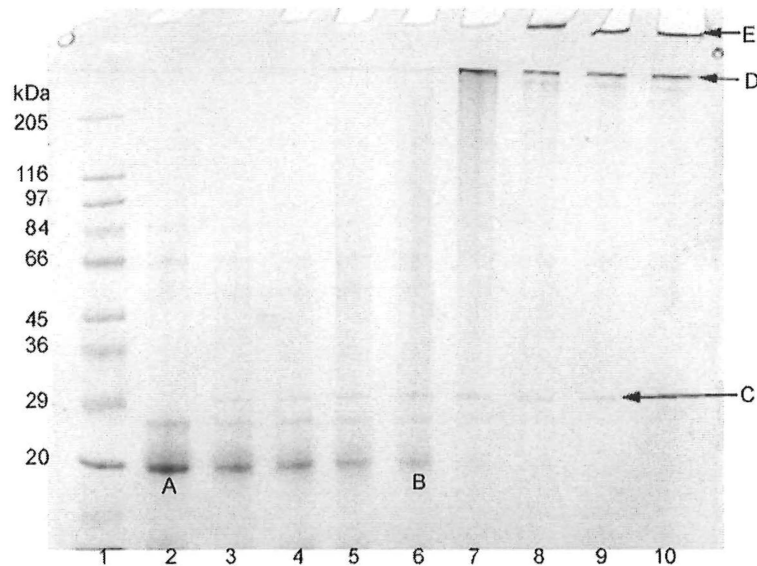


Figure 5.19 (b): SDS-PAGE of κ -casein with TGA [0.05 g / mL], over time. **Lane 1:** Sigmamarker; **Lane 2:** Control; **Lane 3:** incubation time 0 minutes; **Lane 4:** incubation time 5 minutes; **Lane 5:** incubation time 15 minutes; **Lane 6:** incubation time 30 minutes; **Lane 7:** incubation time 45 minutes; **Lane 8:** incubation time 60 minutes; **Lane 9:** incubation time 1.25 hours. **Lane 10:** 1.5 hours.

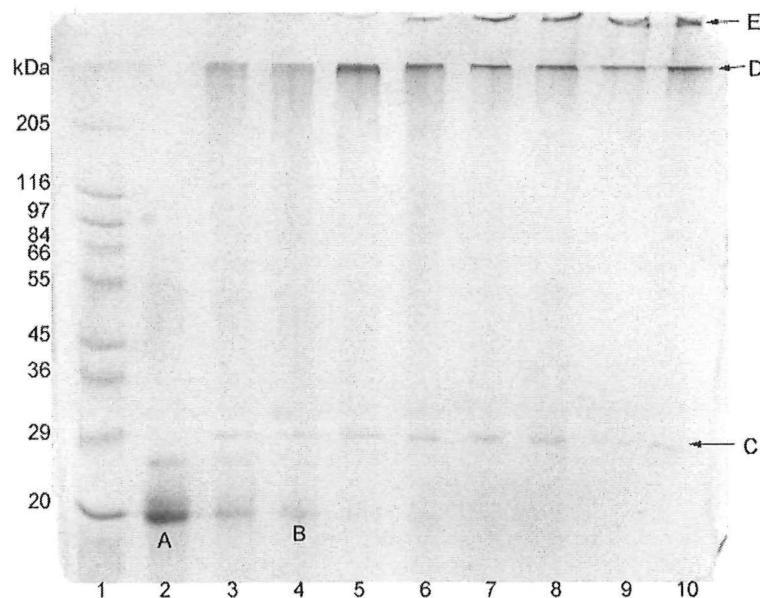
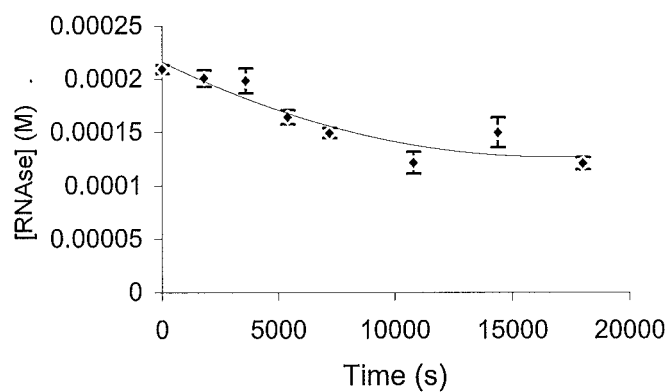
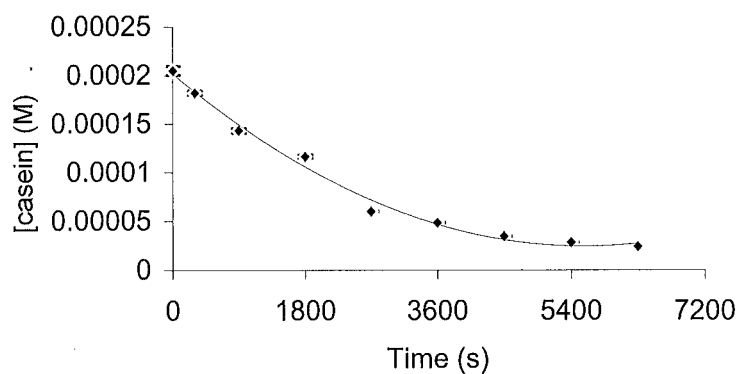


Figure 5.20: Typical reactions of the model proteins with TGA. Error bars represent the standard error of the mean of three measurements.

(a) RNase A



(b) κ -casein



(c) BSA

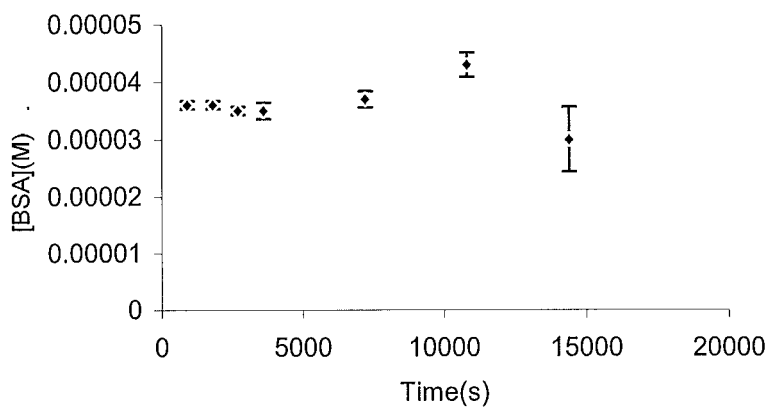
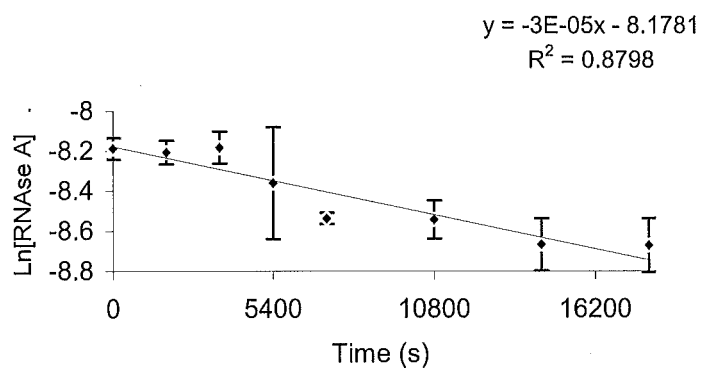
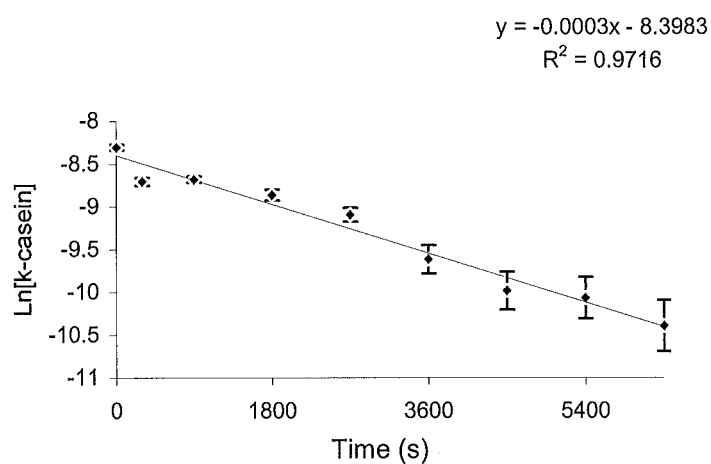


Figure 5.21: Log graphs of model protein concentration over time of incubation with TGA. Error bars represent the standard error of the mean of three measurements.

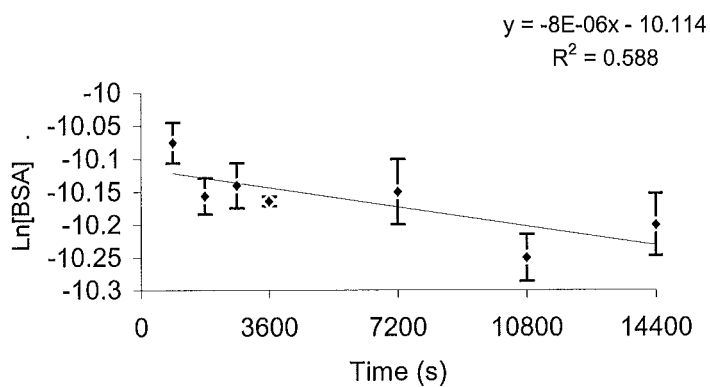
(a) RNase A



(b) κ -casein



(c) BSA



The band labelled C in the gel photographs represents the enzyme, TGA. The crosslinked material can be seen at position D, or at E, which is in the stacking gel. Figure 5.19 (a) shows the results of using a TGA concentration of 0.01 g / mL for the incubation with κ -casein. In this picture, the last band of κ -casein was in lane 6, corresponding to an incubation time of 30 minutes. When the concentration of TGA was increased to 0.05 g / mL, however (figure 5.19 (b)), the last band was seen at lane 4 representing 5 minutes incubation. The accompanying crosslinked products can be seen from lane 3 on this gel.

Protein concentration data from image analyses showed an exponential drop for RNase A and κ -casein, (figures 5.20 (a) and (b)), whereas BSA was confirmed to be essentially unreactive (figure 5.20 (c)).

In order to derive a simple measure of crosslinking rate, the data were plotted on a log scale, as shown in the figures 5.21 (a, b and c).

Based on the logarithmic plots, the relative rates of reaction for the proteins with TGA are shown in table 5.3.

Table 5.3: Relative rates of model protein reactions with TGA.

Protein	Relative rate (m)	R ²
RNase A	3.0×10^{-5}	0.88
κ -casein	3.0×10^{-4}	0.97
BSA	8.0×10^{-6}	0.59

As seen above, the reaction rates in order of increasing magnitude were: BSA < RNase A < κ -casein. κ -Casein also displayed the most accurately reproducible results, as seen by the R² value. In contrast, it proved very difficult to obtain a reliable reaction rate for BSA. A much slower rate than the other two proteins was, however, estimated. These rates for crosslinking by TGA are consistent with the reported descriptions of

these proteins in the literature (Ikura et al, 1984; Wong et al, 1996; Grootjens et al, 1995).

5.4.5 Substrate specificity of TGA for the model proteins

It has been known for some time that a cysteine residue (Cys 314) in the active site of TGA is crucial to the transamidation reaction (Pedersen et al, 1994). This residue is important for the formation of a thio-acyl intermediate. In mammalian TGAs, this cysteine is located in a catalytic triad arrangement with histidine and aspartate residues in the active site. Studies of microbial TGAs suggest that while a cysteine residue is still necessary for activation, this may be located within a variety of different active site sequences, implying that TGA from microbes are quite heterogeneous (Kobayashi et al, 1998; Makarova et al, 1999).

One of the main determinants of specificity is thought to be the nature of the side chains of the amino acids immediately surrounding the active site cysteine residue, as determined by the reduction of activity in site-directed mutagenesis studies (Hettasch and Greenberg, 1994). Thus, particular features in the substrates themselves play an important role in catalysis (Coussons et al, 1992; Grootjans et al, 1995). In particular, in both microbial and mammalian TGA, it has been shown that both the primary structure and total conformation of a protein are important in determining its suitability as a TGA substrate (Coussons et al, 1992; Taguchi et al, 2000).

The results obtained in our studies confirmed that κ -casein was a good TGA substrate. In milk, the caseins exist as stable calcium phosphate-protein complexes termed micelles. κ -Casein is relatively hydrophobic among the caseins, assuming a compact ellipsoid micelle with the nonpolar side chains buried in the interior (Wong et al, 1996). The hydrophilic glutamine and lysine crosslinking sites however, have been detected on solvent-exposed surface regions, by a radioactive site-specific probe, (Christensen et al, 1996).

RNAse displayed a reaction rate approximately ten times slower than κ -casein. The slower rate was attributed to the selectivity of the enzyme towards the glutaminyll residues. Grootjens et al (1995) found TGA to be much more selective towards the glutamine substrates than the lysine donor substrates. It was also reported by Coussons

et al (1992) that the minimal requirements for a glutaminy residue to act as a substrate are, (i) that the glutamine must satisfy an “accessibility” criterion by either being in a highly flexible region of the polypeptide chain or being clearly exposed to the solvent in a more structured region and (ii) that in the sequence surrounding the glutamine there must be an absence of features that appear to discourage correct interaction with the enzyme. The results shown here confirm the contention of these researchers that in RNase A, the accessibility of the glutamine residues is somewhat restricted.

BSA was found to be the least reactive as a TGA substrate. The calculated reaction rate was 100 times as slow as that of κ -casein. This was not surprising, as BSA has a known low reactivity with TGA, having even been used as an inhibitor of the reaction (Taguchi et al, 2000). In spite of the 21 glutamine and 60 lysine residues present, it has been suggested that the globular ellipsoid conformation, similar to ovalbumin, results in these occurring mainly within loops and thus being unavailable on the surface for crosslinking (Ikura et al, 1984; Han and Damodaran, 1996). Several investigations have demonstrated the enhancement of susceptibility of such globular proteins to TGA by chemical modification (Ikura et al, 1984; Larre et al, 1993; Matsumura et al, 1996), but these are often inappropriate in food systems.

Thus, we have developed a successful method for monitoring the progress of the crosslinking reaction. This method is suited to comparing the activities of TGA on different potential substrates and studying the relative extent of crosslinking on specific food protein substrates. On the basis of these results, we applied the protein quantification method to the crosslinking of a high molecular weight glutenin by TGA.

5.5 Crosslinking of a high molecular weight glutenin by TGA

5.5.1 Predicting crosslinking rate for the glutenin subunit

High molecular weight glutenin subunits, although minor in terms of quantity, are key factors in breadmaking, being the major determinants of gluten elasticity (Gianibelli et al, 2001). Gluten proteins are high in glutamine but low in lysine (Wrigley & Bietz, 1988). The studies described so far in this chapter have indicated that not only are the number and positions of the lysine residues significant for crosslinking, but that the

particular glutamine residues that are able to be crosslinked must be in the correct location in the protein.

Since many glutamine residues are present in the glutenins, it is reasonable to expect that there will be several that could fulfil the requirements for crosslinking by TGA. This, as well as the possible lower selectivity of TGA towards the lysine residues (Coussons et al, 1992), explain why the high molecular weight glutenin would become rapidly crosslinked by TGA, in spite of the low lysine content of this protein.

5.5.2 Reaction rate for a HMW subunit of glutenin

To test this, one particular high molecular weight (HMW) subunit was purified from a back-crossed flour variety, known to consist only of subunit 1, using the method of Sutton (1991).

This involved initially extracting the total proteins with a 50% (v/v) solution of 1-propanol containing the reducing agent DTT. This was followed by the precipitation of the HMW glutenins, by increasing the 1-propanol concentration to 60% (v/v) while gently vortexing the solution. The precipitate containing the HMW subunits was pooled and freeze-dried.

This protein was treated in the same way as the model proteins in this chapter, and incubated with an excess TGA concentration. SDS-PAGE and image analyses were carried out as before, to calculate the rate of crosslinking. The HMW glutenin was seen at 84 kDa under reducing conditions. Figures 5.22 (a) and (b) show gels of this reaction.

Figure 5.22 (a) shows the disappearance of the protein band immediately upon addition of TGA. The very rapid nature of the reaction with the HMW subunit meant that, in order to obtain a time-course for the reaction, the enzyme concentration had to be reduced to a sufficiently low level, as close to the threshold as possible. This was finally achieved at a TGA concentration of 0.02 g / mL, and is shown in figure 5.22 (b).

Figure 5.22 (a): SDS-PAGE of the HMW glutenin with [0.1 g / mL TGA], over time. **Lane 1:** Sigmamarker; **Lane 2:** HMW glutenin control; **Lane 3:** incubation 0-time; **Lane 4:** 5 minutes; **Lane 5:** 15 minutes; **Lane 6:** 30 minutes; **Lane 7:** 1 hour; **Lane 8:** 3 hours; **Lane 9:** 5 hours.

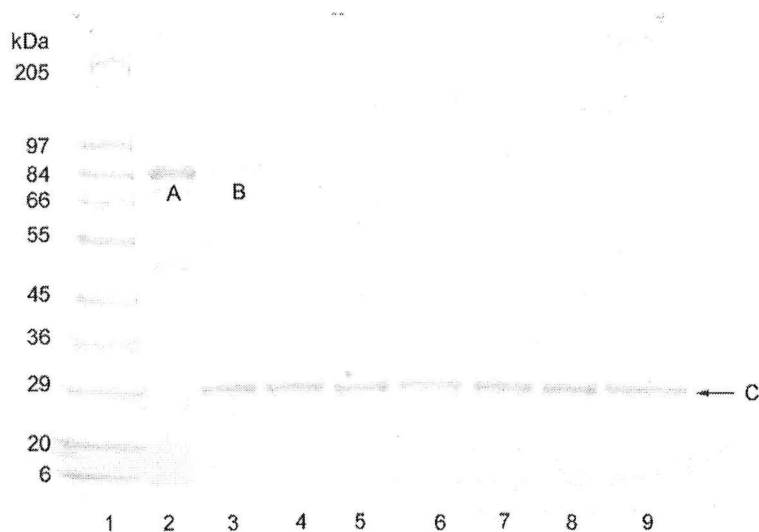
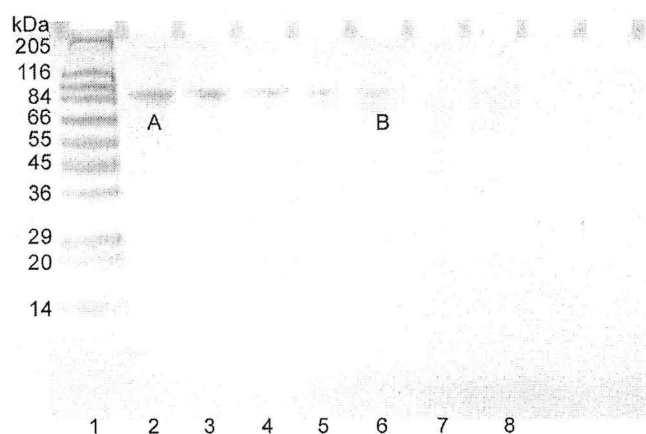
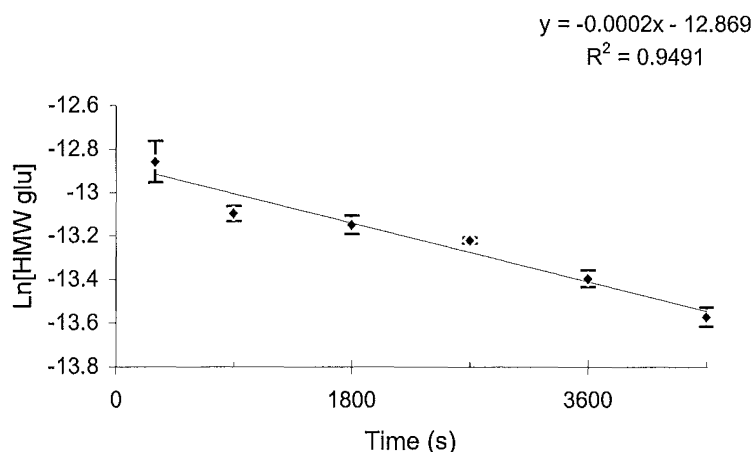


Figure 5.22 (b): SDS-PAGE of the HMW glutenin with TGA [0.02 g / mL], over time. **Lane 1:** Sigmamarker; **Lane 2:** HMW glutenin control; **Lane 3:** incubation 0-time; **Lane 4:** 15 minutes; **Lane 5:** 30 minutes; **Lane 6:** 45 minutes; **Lane 7:** 60 minutes; **Lane 8:** 75 minutes.



Measurements of the rate of the reaction between TGA and the HMW glutenin subunit showed that the relative rate for the HMW glutenin subunit, at 2×10^{-4} (figure 5.23), was extremely rapid, in the order of magnitude comparable to that of κ -casein.

Figure 5.23: Graph representing a typical reaction of the purified HMW glutenin with TGA. Error bars represent the standard error of the mean of three measurements.



The large difference in the lysine content of these two proteins only serves to confirm that which was stated earlier, namely that the number of lysine residues is not nearly as significant as their position on the protein surface. Unlike the gliadins, which were only crosslinked *in vitro*, once the lysine and / or glutamine residues were exposed, the glutenins appear to have their reactive residues on the protein surface, throughout the gluten network, thus serving as readily available targets for the crosslinking action of TGA. Moreover, the few crosslinks that are possible with the low lysine content of the glutenins seem to be sufficient to exert the dramatic, irreversible effects in the protein substrate, and hence, in the baked product.

5.6 Summary

Several assay techniques exist for the measurement of TGA activity. However, not all of these measure the crosslinking reaction itself, but instead measure another TGA-catalysed reaction, namely amine incorporation. The hydroxamate assay was initially adapted for measuring TGA in dough substrates. TGA was found to remain active in the dough, even after proofing and frozen storage. This was followed by investigations

into measurements of lysine levels to indicate TGA activity. A 15% drop was consistently observed. Finally, new methodology was developed in which the crosslinking of proteins by TGA can directly be directly monitored. This method, developed first with model proteins, was then applied to the crosslinking of a HMW glutenin subunit, by TGA. The results were as predicted according to the reported behaviour of TGA with the model proteins, thereby providing a new assay method with which to study the molecular basis of the most significant improvements seen in baked goods, with TGA.

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Chapter Six

SUMMARY AND CONCLUSIONS

The subject of this research was the modification of the proteins of wheat flour by means of the enzymes transglutaminase (TGA) and glucose oxidase. The particular mechanism through which these enzymes act on proteins is *via* protein crosslinking, which has large structural and functional consequences for the polymeric wheat proteins.

In this research, it has been established that the TGA-catalysed reaction took place during the mixing process. There was no further evidence of reaction after this initial phase, either during the proofing of the dough, upon frozen storage of the dough, or with increasing enzyme dosage.

All the proteins in the wheat dough were analysed after reaction with TGA *in vitro* and *in situ*, in the dough. The greatest effect of TGA was on the SDS-insoluble glutenins. The albumins and globulins, and to a more variable degree the SDS-soluble glutenins, were also crosslinked by TGA. Whereas the gliadins became crosslinked by TGA *in vitro*, this was not seen in the dough itself, suggesting that the ability of the gliadins to act as substrates for the enzyme is dependent on their environment, to a much greater extent than the other wheat protein fractions. The dramatic crosslinking effects of TGA are attributed its action on the SDS-insoluble glutenins. This results in an increased dough strength and a substantial increase in pastry volume, an effect which is preserved upon frozen storage.

The effect of glucose oxidase on bread and croissants was found to be much less dramatic, effecting mainly the texture and crumb properties of baked goods, with no dramatic increase in volume of croissants and pastries. At the molecular level, the albumins and globulins were crosslinked, and, to a lesser extent, the glutenins.

Contrary to literature reports, both disulfide and non-disulfide crosslinks were formed by the action of glucose oxidase. It is suggested that the non-disulfide crosslinks are either dityrosine or Maillard type crosslinks.

Thus, at the molecular level, TGA and glucose oxidase were found to bring about quite different changes to wheat proteins. Correspondingly, the macroscopic effects on baked products were also different. We have not sought to attribute this difference in behaviour to the specific chemical nature of the introduced crosslinks, but rather to the relative significance of the particular wheat proteins being crosslinked. An analysis of these results in the context of previous literature supports the conjecture that:

- the crosslinking of the albumins and globulins into larger aggregates facilitates their involvement in dough development, which is translated into improved crumb properties of the baked product;

and

- the crosslinking of the HMW glutenins is responsible for improved dough strength and a marked improvement in pastry volume.

TGA was the more promising of the two enzymes studied for use as a flour improver. Further research was therefore carried out in order to establish methodology with which to monitor its presence within the dough and study the rate of the crosslinking reaction.

Firstly, we explored various methods for monitoring the drop in lysine concentration that occurred upon reaction of TGA with wheat proteins. A drop of available lysine of 15% was consistently seen in croissant dough proteins, as measured by both the OPA and TNBS methods. Consistent with the crosslinking studies, this small, but significant, drop occurred during mixing and remained throughout all subsequent processing steps. Since the lysine within TGA-induced crosslinks is thought to be

nutritionally available, these results suggest that treatment with this enzyme protects 15% of wheat protein lysine residues from nutritional damage during processing.

Monitoring lysine loss did not provide a convenient means of measuring the activity of TGA. A literature assay, the hydroxamate method, was adapted for use in dough and found to provide a simple and convenient means of assaying the activity of TGA in bread and croissant doughs. Using this assay, it was established that TGA remained active during mixing, proofing, and frozen storage. Thus, the lack of reaction after the initial crosslinking in the mixer cannot be attributed to a loss of activity and, instead, suggests a lack of substrate availability.

The hydroxamate assay represents an ideal method to detect the enzyme in commercial dough preparations. However, since it relies on a side activity of TGA – incorporation of hydroxylamine into a protein – it does not provide a means to monitor crosslinking *per se*. In order to study the crosslinking of food proteins directly, a new method was developed using model proteins. Building on the success of our earlier analyses by SDS-PAGE, the assay was based on the analysis of SDS-PAGE gels using image analysis.

The three model proteins chosen for method development were κ -casein, ribonuclease A and BSA, which were reported in the literature to have widely varying crosslinking abilities. Thus, κ -casein displayed the fastest reaction, with ribonuclease A of intermediate reactivity and BSA extremely unreactive. This method represents a valuable new tool with which to embark upon mechanistic studies of the crosslinking of food proteins with TGA. With methodology in hand, a HMW glutenin subunit was then tested and was found to undergo very rapid crosslinking in the presence of TGA, consistent with the earlier results based on total protein extracts.

The removal of potassium bromate from the baking industry has seen the testing of various oxidative enzymes, alone and in combination with other enzymes, to replace its improver effect. These have had limited success. Thus TGA, with its beneficial effects on wheat proteins, promises to be an ideal solution to the problem of strengthening the dough network, without the addition of chemicals. The work

presented in this thesis represents a significant advance in our understanding of the mechanism of action of this enzyme, paving the way for optimisation of its use in industry in a wider context.

Chapter Seven

EXPERIMENTAL

7.1 General Methods

7.1.1 Materials

Unless otherwise stated, all materials were obtained from Sigma Chemical Company Ltd, Aldrich Chemicals or BDH Laboratory Supplies and were generally of analytical grade. Solvents were purchased from BDH and were of analytical grade.

Transglutaminase (TGA) was obtained from Amcor Trading Pty. Ltd, Australia. The commercial preparation consisted of 20% protein and 80% dextrin filler.

Glucose oxidase was obtained from Enzyme Services (New Zealand) Ltd. The batch used was Ensidase GO WS25, with an activity of 20-30 titrimetric units. Each titrimetric unit oxidises 3 mg of β -D-glucose to gluconic acid in 15 minutes at 35°C, pH 5.1.

Bovine serum albumin (BSA) was obtained from Sigma Chemical Company Ltd.

κ -Casein was from Sigma Chemical Company Ltd (C-0406) and was of >80% purity.

Ribonuclease A (RNase A) was Type XII-A (from bovine pancreas) and was obtained from Sigma Chemical Company Ltd.

Commercial bread baking flour was purchased from Champion Flour Mill and was stored at -20°C. WRI fat was obtained from NZ Bakels Ltd and contained 20% sodium stearoyl-2-lactylate, 15% diacetyl tartaric esters of distilled monoglycerides, 20% enzyme active soya (55% flour plus anticaking agent). Saxa plain salt, Chelsea sugar

and Pinnacle yeast from NZ Food Industries Ltd were used. Flour for croissants was Stratus brand.

Fat extractions of croissant and biscuit samples were carried out using a standard laboratory Soxhlet apparatus and Whatman 30 mm x 100 mm extraction thimbles.

High speed centrifugation was performed on an Eppendorf Centrifuge 5403, on a small scale (<1.5 mL) at up to 15000 r.p.m, and on a large scale (> 50 mL) at up to 5000 r.p.m. Low speed centrifugation was achieved using a benchtop microcentrifuge supplied by Qualitron Inc.

Ultraviolet (UV) spectroscopy was carried out on a Hewlett Packard 8452A Diode Array Spectrophotometer interfaced with a personal computer running Hewlett Packard 8452A UV-visible operating software. Alternatively UV absorbance measurements were taken with the Bio-Rad SmartSpec 3000 spectrophotometer.

All pH measurements were performed using an EDT Instruments BA 350 series 3 pH meter fitted with an EDT Instruments E8030 electrode, calibrated against standard buffers at pH 4.0, pH 7.0 and pH 9.0.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was routinely run using a Bio-Rad 300 power pack. Gel boxes and glass plates were provided by technical services of Canterbury University. Mini i-gels were run using a Bio-Rad i-gel kit with pre-cast Gradipore 8% acrylamide or gradient 8-16% acrylamide gels.

High performance liquid chromatographic (HPLC) analyses were carried out using the Waters HPLC system. Water for HPLC was produced with a Milli-Q Water Purification System (Millipore).

Protein quantification of electrophoretic gels was accomplished with the Bio-Rad Quantity One image analysis computer software (Bio-Rad, 2000).

7.1.2 Mechanical dough development method for bread dough preparation

Bread doughs were prepared as for the 125 g mini bake loaves described by Larsen and Greenwood (1991).

Bread dough (control)

flour	125.0 g
salt	2.5 g
WRI fat.....	1.5 g
sugar	0.94 g
water absorption	60%
work input	10 WH /kg

Bread dough (TGA)

flour	125.0 g
salt	2.5 g
WRI fat.....	1.5 g
sugar	0.94 g
TGA 1x.....	5,000 p.p.m
or 2x	10,000 p.p.m
water absorption	60%
work input	10 WH /kg

Bread dough (glucose oxidase)

flour	125.0 g
salt	2.5 g
WRI fat.....	1.5 g
sugar	0.94 g
glucose oxidase	0.003 g
water absorption	60%
work input	11.3 WH /kg

Additives (as required):

H ₂ O ₂ (10% solution).....	3.4 mL
glucose.....	0.5%

Flour, salt, fat and any additives were weighed into a metal tin. Water (80 mL) at approximately 10°C was poured into a measuring cylinder. The weighed materials were added to the mixing bowl of a variable speed Mitchell 1000 Electronic Dough Developer, followed by the water containing the additives as required. Doughs were mixed at a constant speed of 150 r.p.m. The mixing curve of each dough was monitored and the mixer turned off when the optimum degree of mixing (11.3 WH/kg) had been reached. The dough was then removed from the mixer, frozen in liquid nitrogen and stored at -10°C.

7.1.3 General procedure for the preparation of croissant doughs***Croissant dough (control)***

flour.....	1111.11 g
yeast.....	44.45 g
salt.....	20.37 g
fat (lard).....	22.22 g
sugar.....	22.22 g
water.....	660.00 g

Croissant dough (TGA)

flour.....	1111.11 g
yeast.....	44.45 g
salt.....	20.37 g
fat (lard).....	22.22 g
sugar.....	22.22 g
water.....	660.00 g
TGA (5000 p.p.m).....	5.56 g

Croissant dough (glucose oxidase)

flour	1111.11 g
yeast.....	44.45 g
salt	20.37 g
fat (lard).....	22.22 g
sugar	22.22 g
water	660.00 g
glucose oxidase (10 p.p.m).....	5.56 g

Additives (as required):

H ₂ O ₂ (10% solution)	3.4 mL
glucose.....	0.5%

Dry ingredients at 8°C were mixed on a Baker Perkins mixer, fitted with a temperature-controlled water jacket and cooled with ice, at 86 r.p.m for 2 minutes. The lard, yeast and iced water were added and mixed for a further 2 minutes. The dough was rested, covered at 6°C for 5 minutes before lamination on a SINMAG pastry brake, at 17°C, using butter sheets (459 g). Pastries were then processed at 12°C. The pastry sheet was cut into triangles with a sharp knife to a standard size (length 120 mm, width 135 mm, weight 62 g). Each triangle was reduced to 2.4 mm on a hand driven pastry brake, then formed into croissants. Croissants were either proofed at 23°C or samples taken from the croissants were frozen in liquid nitrogen. Croissants prepared for the freezing trials were frozen at -20°C.

7.1.4 General procedure for the preparation of biscuit doughs***Biscuit dough***

flour	385.0 g
salt	4.8 g
shortening.....	160.0 g
sugar (white).....	128.0 g
sugar (brown)	128.0 g
eggs	105.0 g
NaHCO ₃	3.2 g
TGA (for treated biscuits)	1.9 g

Biscuit doughs were mixed in an electric Hobart mixer with a 3 L bowl and a flat beater. Biscuit doughs were rolled to 7 mm thick and cut with a cookie cutter of 60 cm internal diameter. Dough samples were made in duplicate for analysis.

7.1.5 Procedure for the extraction of fat from croissant doughs

To remove the fat from the croissant and biscuit doughs, samples were freeze dried and ground using a mortar and pestle. A portion (5 g) of each sample was weighed into an extraction thimble, which was then sealed with a cotton wool plug, and placed in a labelled glass extractor. Boiling chips and n-hexane (250 mL) were added to round bottom flasks in a fumehood, and placed on a series of heating elements of a standard Soxhlet apparatus. After approximately 23 cycles, the water and heating elements were turned off. After cooling, the apparatus was dismantled and the thimbles placed into labelled plastic bags. The bags were placed under vacuum to remove hexane from the samples. The samples were then placed into labelled containers, ready for protein extraction. The waste hexane containing the extracted fat was tested for the presence of nitrogen and found to contain negligible amounts, indicating that protein was not being lost in the fat extraction process.

7.2 Extraction of dough proteins

7.2.1 Extraction of proteins

The routine extraction methods used were based on a modification of the methods of Hay and Sutton (1990) and Batey et al (1991). Doughs of bread or croissants were freeze-dried and ground into a flour-like powder. Fat extraction was carried out on croissant or biscuit doughs. 100 mg samples from each bread, croissant and biscuit dough were weighed into 1.5 mL Eppendorf tubes.

Albumins and globulins

0.4 mL of 2% (w/v) sodium chloride was added to the 100 mg samples and vortex mixed every 5 minutes for 30 minutes. Samples were centrifuged (10,000 g, 5 minutes) and the clarified supernatant was transferred to a new Eppendorf tube and stored at -10°C prior to analysis by SDS-PAGE and HPLC.

Gliadins

The residual pellet from above was resuspended in 70% w/v aqueous ethanol (0.4 mL) with vortex mixing every 5 minutes for 30 minutes. The sample was then centrifuged (10,000 g, 5 minutes) and the supernatant was transferred and stored at -10°C until further analysis.

SDS-soluble glutenins***0.5% SDS- 0.05 M phosphate buffer, pH 6.9***

Na₂HPO₄ 3.53 g / 500 mL

KH₂PO₄ 3.39 g / 500 mL

The above solutions were mixed together to pH 6.8 and 5.0 g SDS was added. The mixture was stirred and gently heated in a microwave oven until completely dissolved.

SDS-soluble glutenins were extracted by resuspending the residual pellet in 0.4 mL of the above SDS buffer (for 30 minutes with vortex mixing every 5 minutes. After centrifugation (10,000 g, 5 minutes) the clarified supernatant was removed and stored at -10°C.

SDS-insoluble glutenins

The residual pellet was resuspended in the same SDS buffer as above and sonicated at 90-100 W for 2 minutes using a Branson sonic disrupter. The sample was then vortex mixed, centrifuged and the supernatant removed and stored along with the other fractions.

7.3 Dough protein analysis**7.3.1 Preparation of SDS-PAGE**

The following is a modification of the method described in Fayle (1998). Analysis of proteins by SDS-PAGE was a routine procedure, ensuring multiple sets of results for each protein fraction.

Preparation of stock solutions**Acrylamide monomer solution***(29.2% acrylamide; 0.8% bis-acrylamide)*

acrylamide 116.8 g

bis-acrylamide 3.2 g

dH₂O to 400 mL

stored in a dark bottle at 4°C

Resolving gel buffer*(1.5 M Tris-HCl pH 8.8)*

Tris-HCl 35.3 g

dH₂O to 200 mL

The solution of Tris-HCl was prepared and the pH was adjusted to 8.8. The buffer was stored at 4°C

Stacking gel buffer*(0.5 M Tris-HCl pH 6.8)*

Tris-HCl 3.0 g

dH₂O to 50 mL

The solution of Tris-HCl was prepared and the pH was adjusted to 6.8. The buffer was stored at 4°C.

2x Treatment buffer (gel loading buffer)

1 M Tris-HCl pH 6.8 125 µL

10% (w/v) SDS 2.0 mL

glycerol 1.0 mL

2-mercaptoethanol 500 µL

1% (w/v) bromophenol blue 125 µL

dH₂O 750 µL

stored at -10°C

0.1% Coomassie brilliant blue stain

Coomassie brilliant blue.....	0.1 g
glacial acetic acid	10 mL
methanol.....	50 mL
dH ₂ O.....	40 mL

The was mixed thoroughly for 30 minutes, then filtered and stored at room temperature

Destain

(5% methanol; 10% acetic acid)

glacial acetic acid	100 mL
methanol.....	50 mL
dH ₂ O.....	850 mL

5x Tank buffer

Tris	45.0 g
Glycine	216.0 g
SDS	15.0 g
dH ₂ O to 3000 mL	
stored at 4°C	

Preparation of SDS-PAGE gels

Table 7.1: Components for preparation of 7% SDS-PAGE gels.

Component	Resolving gel (7.0% acrylamide, 0.19% bis-acrylamide)	Stacking gel (3.5% acrylamide, 0.1% bis-acrylamide)
Acrylamide monomer	6.0 mL	1.33 mL
Resolving gel buffer	6.25 mL	-
Stacking gel buffer	-	2.5 mL
dH ₂ O	12.3 mL	6.1 mL
10% SDS	250 µL	1.0 mL
10% AMPS	125 µL	50 µL
TEMED	8.3 µL	25 µL
Final volume	25.0 mL	11.0 mL

Table 7.2: Components for preparation of 12% SDS-PAGE gels.

Component	Resolving gel (12.2% acrylamide, 0.33% bis-acrylamide)	Stacking gel (3.5% acrylamide, 0.1% bis-acrylamide)
Acrylamide monomer	10.4 mL	1.33 mL
Resolving gel buffer	6.25 mL	-
Stacking gel buffer	-	2.5 mL
dH ₂ O	7.90 mL	6.1 mL
10% SDS	250 µL	1.0 mL
10% AMPS	125 µL	50 µL
TEMED	8.3 µL	25 µL
Final volume	25.0 mL	11.0 mL

Two glass plates and two Teflon 1.5 mm spacers were washed with Jif liquid and dried. They were then cleaned further with 1-propanol and dried with paper towels. The glass plates were aligned together with the spacers in between them. They were clamped onto the gel box, into which a moistened layer of filter paper had been placed along the base. A heated 2% agarose solution was then pipetted down the inside edges of the glass sandwich, to prevent leakage of the gel solution.

The resolving and stacking gels were prepared according to the recipe above. Acrylamide monomer solution was transferred to a clean, dry vacuum flask. The mixture was degassed under vacuum for 20 minutes, until bubbles ceased to appear. The 10% SDS, TEMED and freshly prepared. AMPS solution were added to the degassed gel solution and gently swirled to mix. The gel solution was slowly poured in between the glass plates, leaving a space of approximately 4.0cm at the top, into which the stacking gel would later be poured. 1.0 mL of distilled water was pipetted onto the top of the resolving gel, which was left to polymerise overnight.

Once the resolving gel had set, the distilled water was poured off. The stacking gel was made in the same way as the resolving gel and poured into the remaining gap over the resolving gel. A Perspex well comb was immediately inserted into the stacking gel

mixture ensuring no air bubbles were present, and the assembly was left for approximately 45 minutes to polymerise at 4°C.

After polymerisation, both reservoirs of the gel box were filled with tank buffer. The well positions were marked and the comb was carefully removed, exposing the sample wells. Leads from a power pack were connected to the electrodes from the gel box. Each of the prepared protein samples and markers was carefully loaded into a separate well. The load volume necessary for obtaining clear visible bands was 20 µL for albumin and globulin fractions, and 45 µL for glutenins. The samples were electrophoresed at a constant current of 30 mA, 100 V (for two gels 40 mA, 194 V) until the bromophenol dye contained in the sample treatment buffer reached the bottom of the gel, approximately 5 hours.

At the end of this time, the power was turned off, the gel apparatus dismantled and the glass plates carefully prised apart. The gel was submerged in Coomassie brilliant blue stain overnight at room temperature, before being destained. The destain solution was regularly changed to remove excess stain. The gel could then be stored and photographed.

7.3.2 Commercially prepared SDS-PAGE gels

SDS-PAGE was also conducted using Tris-glycine pre-cast *i*-gels. The gels were placed in the gasket of the Bio-Rad SDS-PAGE gel rig, which was lowered into the plastic trough. Sufficient tank buffer was poured into the inner and outer compartments of the system. A pasteur pipette was used to thoroughly rinse out the wells prior to sample loading. Between 5 and 50 µL sample was loaded into each of the wells. The rig was then covered and connected to a power pack. Electrophoresis was carried out at a constant voltage of 150 V, until the loading dye had reached the bottom of the gel. At the end of this time, the gels were removed from their covers and stained with Coomassie brilliant blue stain for one hour. They were subsequently destained and photographed.

7.3.3 Preparation of protein samples for electrophoresis

Each of the extracted protein fractions and Sigmamarker, a wide range molecular weight standard, was thawed and an aliquot (50 μ L of the fractions and 10 μ L of the marker) was placed in a labelled Eppendorf tube. An equivalent volume of reducing 2x treatment buffer was added to each sample and the mixture gently vortexed. Samples were placed in a boiling water bath for 3 minutes to help solubilise the samples and to aid reduction of disulfide bonds. The samples were centrifuged at 10,000 g for 5 minutes, prior to loading onto the gel. For the preparation of non-reduced samples, the treatment buffer used was made without the reducing agent, mercaptoethanol.

Table 7.3: Sigma marker molecular weight distribution.

Proteins	Mol. Wt. (kDa)
Rabbit muscle myosin	205
<i>E.coli</i> β -galactosidase	116
Rabbit muscle phosphorylase b	97
Rabbit muscle fructose-6-phosphate kinase	84
Bovine serum albumin	66
Bovine liver glutamic dehydrogenase	55
Ovalbumin	45
Rabbit muscle glyceraldehyde-3-phosphate	36
Bovine erythrocyte anhydrase	29
Bovine pancreas trypsinogen	24
Soybean trypsin inhibitor	20
Bovine milk α -lactalbumin	14
Bovine lung aprotinin	7

7.3.4 Analysis by HPLC

Protein fractions were extracted as described in section 7.2.1. 20 μ L of each extract was injected onto the HPLC columns described below. The procedures were conducted twice under identical conditions.

SE-HPLC

Size-exclusion HPLC conditions were based on the method of Gupta et al (1993). A Waters W2690 solvent delivery/ control/ sample injector system and a Waters 490 UV-visible detector were used. The column was a BioSep SEC-4000S (300 x 7.5 mm) with a guard column (75 x 7.5 mm). The solvents used were (A) water containing 0.1% (v/v) TFA and (B) acetonitrile containing 0.1% (v/v) TFA. Deaeration was achieved by vacuum filtration through a 0.22 μ m filter, rapid sparging with helium (100 mL/minute for 10 minutes) and constant slow bubbling of helium into capped, vented solvent reservoirs (30 ml/minute). Samples (20 μ L) were injected onto the column, which was maintained at 20°C and proteins were eluted isocratically at a solvent composition of 50% A: 50% B for 35 minute at a flow rate of 0.5 mL / minute. Eluted components were detected at 210 nm and the chromatographic traces were recorded on a personal computer using the Waters “Millennium” software package.

RP-HPLC

Reversed-phase HPLC conditions were carried out on a Waters W2690 solvent delivery/control/sample injector system and a Waters 490 UV-visible detector. The column was a 150 x 4.6 mm Zorbax 300SB-C8 guard column. Solvents used were (A) water containing 0.1% (v/v) TFA and (B) acetonitrile containing 0.1% (v/v) TFA. Deaeration was achieved by vacuum filtration through a 0.22 μ m filter, rapid sparging with helium (100 mL/min for 10 minutes). Samples (20 μ L) were injected onto the column which was maintained at 50°C, and proteins were eluted using a solvent gradient (23-48% B over 50 minutes, plus a 5 minute hold at 48% B) at a flow rate of 1.0 mL/minute. The column was returned to the initial solvent composition over 1 minute and re-equilibrated for 10 minutes prior to the next analysis. Eluted components were detected at 210 nm and the chromatographic traces were recorded on a personal computer using the Waters “Millennium” software package.

7.4 The effect of variables on the TGA-mediated crosslinking reaction

7.4.1 Effect of TGA concentration on bread dough proteins

Three bread doughs were prepared, a control with no TGA added, one with 5,000 p.p.m TGA added and one with 10,000 p.p.m TGA added. The doughs were frozen in liquid nitrogen immediately upon mixing and freeze-dried. Dough proteins were extracted according to the method in section 7.3.1. The protein fractions were analysed by SDS-PAGE under reducing conditions. the experiment was repeated on at least five different occasions.

7.4.2 Effect of proving time

Bread

Two bread doughs, one treated with TGA and one control were prepared as described in section 7.1.2. A sample of approximately 5 g was taken from each dough and placed in liquid nitrogen immediately upon removal from the mixer. The remaining doughs were left to prove at room temperature for up to 30 minutes, during which time 5 g samples were taken from each dough at 5 minute intervals and placed in liquid nitrogen. At the end of 30 minutes, samples were freeze-dried and finely ground with a mortar and pestle. Protein fractions were extracted and subsequently analysed by SDS-PAGE and HPLC. These procedures were repeated twice to ensure reproducibility.

Croissants

Two croissant doughs, one treated with TGA and one control were prepared as described in section 7.1.3. A sample of approximately 5 g was taken from each dough and placed in liquid nitrogen immediately upon removal from the mixer. The remaining doughs were left to prove at room temperature for up to 30 minutes, during which time 5 g samples were taken from each dough at 5 minute intervals and placed in liquid nitrogen. At the end of 30 minutes, samples were freeze-dried and finely ground with a mortar and pestle. Fat extraction was carried out by the Soxhlet method, and protein fractions were extracted and subsequently analysed by SDS-PAGE and HPLC. These procedures were carried out twice.

7.4.3 Effect of frozen storage on croissants

The croissant trials were conducted twice, under identical conditions over two separate 3 month periods. Croissants were prepared as described in section 7.1.3, with or without 5000 p.p.m. TGA. Ten croissant doughs were prepared, 5 containing TGA and five controls for each of the following freezing time intervals:

Day 0: unfrozen

Day 1: 1 day in frozen storage

Day 7: 7 days in frozen storage

Day 30: 30 days in frozen storage

Day 90: 90 days in frozen storage

All except the day 0 croissants were placed in frozen storage at -20°C . At the end of each time interval, the croissants were removed from the freezer. Upon removal, 5 g samples from representative croissant doughs from each group were either submerged in liquid nitrogen immediately or proofed 50 minutes prior to freeze-drying and fat and protein extraction. The remaining croissants were baked for comparison of quality parameters. The unfrozen croissants from day 0 were also treated in the same manner.

7.5 The reaction of TGA with dough proteins *in vitro*

To examine the reaction of TGA with dough proteins in an *in vitro* buffer system, three control bread or croissant doughs were prepared as in sections 7.1.2 and 7.1.3. Approximately 5 g samples were taken from each dough and submerged in liquid nitrogen. All samples were freeze-dried, and fat was extracted from croissant doughs. Albumins / globulins, gliadins, SDS-soluble and insoluble glutenins were extracted from each of these doughs. At least five separate incubations and analyses were carried out.

Each of the dough protein extracts (15 mg) was added to separate Eppendorf tubes containing 0.1 M Tris-HCl (pH 6.8) solution with 10 mM DTT (750 μL). Each slurry was mixed vigorously to dissolve as much protein as possible. A 65 μL aliquot was removed from each tube and stored as the control at -10°C . A volume (95 μL) of

freshly prepared TGA stock solution was added to each tube, which was vortexed for 30 seconds before being placed in an incubator at 37°C with constant agitation. An aliquot (65 µL) was removed from each tube at 5, 10, 15, 30 and 60 minutes and at 24 hours and stored at -10°C prior to analysis by SDS-PAGE.

7.6 Determination of the lysine content of dough proteins

7.6.1 Sample preparation

To measure the lysine concentration of dough proteins, 3 control bread or croissant doughs were prepared as in sections 7.1.2 and 7.2.2. Approximately 5 g samples were taken from each dough and submerged in liquid nitrogen. All samples were freeze-dried, and fat was extracted from croissant doughs. Albumins / globulins, gliadins, SDS-soluble and insoluble glutenins were extracted from each of these doughs.

To 0.1 g of each fat extracted croissant dough or bread dough 400 µL extracting solution was added. After vortex mixing, the samples were placed in a 60°C water bath for 30 minutes with vortex mixing every 10 minutes. Samples were then centrifuged (10,000 g, 5 minutes) and the supernatant tested for lysine content either by the OPA method or by the TNBS method.

7.6.2 Modified o-phthaldialdehyde (OPA) method for determining lysine availability (Bertrand-Harb et al, 1993; modified: Reid, 1999)

The OPA solution was prepared as follows:

0.1M bicine pH 9.4.....	25 mL
OPA (dissolved in 1 mL methanol)	40 mg
β- mercaptoethanol.....	100 µL
made up to 50 mL with 1- propanol	

Freshly prepared L-lysine standards, at concentrations between 0.01 mg/mL and 0.2 mg/mL were used to established calibration curves with each new batch of OPA solution. Albumin and globulin, gliadin, SDS-soluble and insoluble glutenins were tested as described below. Albumin and globulin samples were diluted 1 in 10 in order

to give results within linear range of the standard curve. Multiple runs were conducted of all samples tested by this method.

To assay lysine availability, a 50 μL aliquot containing 2 mg/mL protein was added to 1.0 mL of OPA reagent in a 1 mL cuvette. The solution was mixed using a pipette tip and incubated for 2 minutes at room temperature. The absorbance was then read against the OPA solution as the blank, at 340 nm.

Determination of protein concentrations

Protein concentrations were determined for expressing the lysine values as mg lysine / mg protein for each extract. Wheat protein concentrations were estimated using a modification of the method of Bradford (1976). A standard curve was established using bovine serum albumin at concentrations between 0.005 mg/mL and 0.03 mg/mL. Samples were diluted if necessary in order to fall in the linear range of the standard curve. 300 μL of each sample was added to a cuvette containing 500 μL of distilled water. 200 μL of Bio-Rad Bradford reagent was added and the contents of the cuvette mixed with an autopipette. The solution was incubated at room temperature for 6 minutes and the absorbance read at 595 nm against a distilled water blank.

7.6.3 Trinitrobenzenesulphonic acid (TNBS) method for lysine determination

The TNBS method for estimating free lysine was based on the method of Cayot and Tainturier (1997). A 0.3% solution of the TNBS reagent in distilled was initially prepared. Freshly prepared L-lysine standards, at concentrations between 0.005 mg/mL and 0.05 mg/mL were used to establish calibration curves with each new batch of TNBS solution. The test solution was prepared as follows:

Protein extract (0.005-0.05 mg/mL).....	1.0 mL
4% NaHCO_3 pH 8.5	1.0 mL
0.1% TNBS in dH_2O	1.0 mL

The above solution was incubated in a 40°C water bath for 2 hours. Following incubation the following solutions were added:

10% SDS 1.0 mL
1 M HCl.....0.5 mL

The samples were thoroughly vortex mixed and the absorbance was read at 344 nm against a blank solution prepared as above except containing 1.0 mL distilled water instead of the protein solution. Multiple runs were carried out to measure lysine content by this method.

7.6.4 Method for extracting combined dough proteins for lysine determination (section 5.3.2)

The following experiment was conducted twice. Three bread doughs were prepared as in 7.1.2. A 5g sample was removed from each and submerged in liquid nitrogen. All samples were freeze-dried and ground to a fine powder.

Following grinding, the following extracting solution was used:

1- propanol50 mL
dH₂O.....50 mL
DTT 1.0 g

To 100 mg of dough, 400 µL of the above solution was added. The mixture was incubated in a 60°C water bath for 30 minutes, with vortexing every 5 minutes. Centrifugation was carried out at 10,000 g, 10 minutes and the supernatant was tested by the OPA method. Five readings were taken in each case.

7.7 TGA activity measurement

7.7.1 Hydroxamate assay for the measurement of transglutaminase activity

The hydroxamate assay was repeated at least 20 times, to perfect the procedure and ensure reproducibility. The following solutions were prepared and the volume shown was placed in a spectrophotometer cuvette:

Solution	Prepared volume	Volume in cuvette
glutathione 50 mM	23.05 mg /1.5 mL	75 μ L
Hydroxylamine 500 mM	52.12 mg /1.5 mL	75 μ L
Tris- acetate 1 M	3.03 g Tris base in 20 mL dH ₂ O; pH 6.0 volume to 50 mL	75 μ L
CBZ-glutaminyI glycine 500 mM	0.06 g / 375 μ L	375 μ L
CaCl ₂ 50 mM	5.5 mg / 1 mL	37.5 μ L
dH ₂ O (for blank) TGA (for sample)	0.5 g /5 mL	112.5 μ L

Ferric chloride solution:

Equal volumes of:

3 M HCl3 mL conc HCl /10 mL dH₂O

5% FeCl₃.6H₂O0.5 g 5% FeCl₃.6H₂O /10 mL 1 M HCl

12% CCl₃COOH1.2 g CCl₃COOH / 10 mL dH₂O

The solution shown in the table was incubated for 5 minutes at 37°C. 0.75 mL of the ferric chloride solution given above was added, the cuvette mixed by inversion and the absorbance read at 525 nm against the prepared blank.

Testing of bread and croissant doughs for TGA activity

The activity of TGA was measured in control and TGA-treated doughs of bread and croissants which were in frozen storage (-20°C) for 8 months. A control and two treated doughs were treated for each product. The procedure was repeated twice. Each time, an aqueous extract was prepared from each freeze-dried and ground (and fat-extracted) dough by using a 1:3 proportion of dough to distilled water, thorough mixing on a vortex mixer for 15 minutes, and centrifugation at 10,000 g for 10 minutes. The supernatant was then measured for TGA activity by the hydroxamate assay.

7.8 Glucose oxidase effects on bread and croissants

7.8.1 Preparation of glucose oxidase bread doughs

Bread doughs were made according to the MDD method outlined in section 7.1.2. Doughs were made in duplicate for each of the following treatments:

Treatment 1: Control

Treatment 2: Control + 0.5% glucose

Treatment 3: glucose oxidase 5.56 g

Treatment 4: glucose oxidase + 0.5% glucose

Treatment 5: 3.4 mL H₂O₂ (10% solution)

Following dough preparation, 5 g samples were taken from each dough and suspended in liquid nitrogen. Samples were then freeze-dried and ground. Protein fractions were extracted as in section 7.2.1 and analysed by SDS-PAGE. Reduced samples were prepared by using the standard treatment buffer containing mercaptoethanol, while samples for non-reduced gels were prepared with the treatment buffer made without the addition of mercaptoethanol. To analyse the baked loaves, two further doughs were made with each of the above treatments and baked. Following mixing, the doughs

were removed and their temperatures recorded. They were then mechanically moulded and enclosed in metal pattie tins before placing in a proving oven at 32°C for 8 minutes. The doughs were then placed in a rectangular baking tin of dimensions 68 mm x 68 mm x 66 mm and baked at 210°C for 45 minute. After baking, the loaves were removed and cooled, prior to analysis of texture and volume.

7.8.2 Preparation of glucose oxidase bread dough for HPLC analysis

Bread doughs were made according to the MDD method outlined in section 7.1.2. Doughs were made in duplicate for each of the following treatments:

Treatment 1: Control

Treatment 2: glucose oxidase 5.56 g (non-prooved)

Treatment 3: glucose oxidase 5.56 g (prooved 30 minutes 23°C)

Treatment 4: glucose oxidase + 0.5% glucose (non-prooved)

Treatment 5: glucose oxidase + 0.5% glucose (prooved 30 minutes 23°C)

Approximately 5 g samples were taken from the doughs and submerged in liquid nitrogen. the proteins were extracted with a 2% salt solution at a proportion of 0.1 g dough / 1 mL solvent. Samples (20µL) were injected onto the SE-HPLC column as in section 7.3.4, and eluted isocratically. Chromatographic traces and peak areas were recorded on a personal computer.

7.8.3 Preparation of glucose oxidase croissant doughs

Croissants were prepared as in section 7.1.3. Duplicate samples were prepared with the following treatments:

Treatment 1: Control

Treatment 2: Control + 0.5% glucose

Treatment 3: glucose oxidase 5.56 g

Treatment 4: glucose oxidase + 0.5% glucose

Following dough preparation, croissants were prepared as described in section 7.1.3. The baked croissants were cooled for at least 1 hour, then weighed and scored for quality parameters.

7.8.4 Crosslinking of a model protein with glucose oxidase

A commercial (Sigma) ovalbumin preparation was used for this experiment. The following procedure was repeated three times, in order to ensure reproducibility. 100 mg of the ovalbumin was weighed into an Eppendorf tube and dissolved in 1.0 mL distilled water. 30 μ L aliquots were dispensed into separate Eppendorf tubes and placed in a water bath at 37°C. Samples for incubation were prepared in triplicate. 0.02 g of glucose oxidase was dissolved in 1.0 mL distilled water. Treatment buffer was added to two of the tubes, which were labelled as controls and placed on ice. 30 μ L of glucose oxidase solution were added to the remaining tubes. These were left in the water bath and removed at various times: 5 minutes, 30 minutes, 1, 2 and 3 hours. Upon removal from incubation, 20 μ L treatment buffer was added and the tubes placed on ice. Samples were then analysed by SDS-PAGE.

The enzyme glucose oxidase was also run separately on SDS-PAGE. A separate solution of enzyme (0.02 g / mL) was prepared with distilled water. 10 μ L of the solution was placed in two Eppendorf tubes. An equivalent volume of treatment buffer containing mercaptoethanol was added to one tube, while an equivalent volume of treatment buffer without mercaptoethanol was added to the second tube. 5 and 10 μ L of each were analysed on 8% pre-cast SDS-PAGE gels.

7.9 Crosslinking of model proteins by TGA

7.9.1 Preparation of the enzyme and substrate solutions

TGA

The required amount of enzyme was weighed into an Eppendorf tube and made up to 1.0 mL with distilled water. The solution was incubated in a 37°C water bath for 30 minutes, with vortex mixing every 5 minutes. Centrifugation was carried out at 10,000 g for 10 minutes, following which the supernatant was removed and stored at 4°C for

the duration of the experiment. TGA was freshly prepared in this manner for each run of model protein incubations.

BSA, RNase, κ -casein

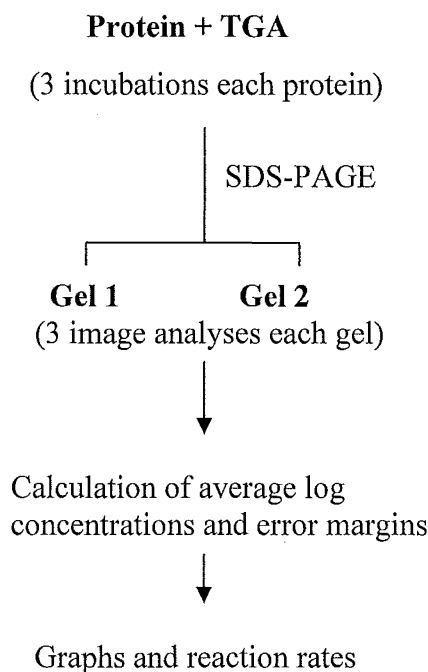
Each of the model proteins was initially run on SDS-PAGE to determine their positions relative to the standard Sigmamarker. Several different concentrations were run to determine the most suitable concentration in each case, namely the concentration required for the production of clear, quantifiable bands. The most suitable concentrations of the model proteins were determined at 0.365mM for RNase A, 0.263 mM for κ -casein and 0.075mM for BSA. Once this was established, the appropriate amount of protein was weighed into an Eppendorf tube. A solution was prepared by making up the weighed protein to 1.0 mL with 0.1M Tris buffer pH 6.8, containing 10 mM DTT to keep the proteins in solution. The solutions were incubated in a 37°C water bath for 30 minutes, with vortex mixing every 5 minutes.

7.9.2 The reaction of TGA with BSA, RNase and κ -casein

The following procedures are modified from Fayle et al (2000). 30 μ L of the protein solution was placed into each of several Eppendorf tubes and incubated in a 37°C water bath for 30 minutes. The appropriate enzyme concentration was also incubated alongside in the same water bath to ensure the same incubation conditions. At the end of 30 minutes, one tube was removed and 30 μ L of the reducing agent mercaptoethanol was added to its contents. This was labelled as the control, placed on ice and subsequently transferred to a -20°C freezer. 30 μ L of the prepared enzyme solution was added to each of the remaining tubes and incubation carried out for the required length of time. Thorough vortex mixing of the tubes was carried out at regular intervals. At the end of each incubation period, 30 μ L of mercaptoethanol was added to quench the reaction. The samples were mixed, placed immediately on ice and transferred to -20°C for storage until analysis by SDS-PAGE.

The samples were subsequently thawed and thoroughly vortex mixed. Following boiling, 5 μ L of each was loaded into a separate well of a prepared 12% polyacrylamide gel. SDS-PAGE was carried out as described previously.

To ensure reproducibility, the following procedure was followed:



7.9.3 Image analysis of the reaction between model proteins and TGA

Gels conducted of each incubation were photographed and image analysis was performed using the Bio-Rad Quantity One image analysis computer software (Bio-Rad, 2000). Following measurements of molecular weight and R_f values for each band of interest, lane traces showing the intensities of the bands in the form of peaks were plotted for each lane to verify that the correct lanes and bands were being detected.

This was then followed by the numerical quantification of the concentration of protein in each band, using the 'volumes' function. Volume analysis takes advantage of the three-dimensional data, consisting of signal intensity, as well as vertical and horizontal positions (BioRad, 2000). To measure concentrations on a gel image, firstly a set area was defined around the band in the control lane. This set area was then also defined around the relevant bands in all the other lanes. Background interference automatically was defined and subtracted from the analysis on each gel. Measurements were then taken relating to the amount of protein in each band.

Although several parameters could be measured using the 'volumes' tool, for our purposes we selected density / mm² (ODu / mm²). Three analyses were performed on each image. The ODu measurements were converted relative to the concentration of the control band. Log values and averages were then calculated and graphed against time for each incubation. Margins of error were calculated for each set of three measurements. The logarithmic slope for each set of data was taken to be representative of the reaction rate in each case.

7.10 Crosslinking of a high molecular weight glutenin subunit by TGA

7.10.1 Purification of a high molecular weight glutenin subunit

A back-crossed variety of wheat flour known to contain a single high molecular weight subunit was selected as the source of glutenin. The subunit was subunit 1, using the nomenclature of Payne et al, 1971. The following purification procedure based on the method of Sutton (1991) was carried out, prior to the reaction of the purified subunit with TGA. The purification was carried out twice, and the reaction with TGA was repeated 4 times, to ensure reproducibility.

100 mg samples of the selected flour were weighed out into six 1.5 mL screw-capped polyethylene centrifuge tubes. Total proteins were initially extracted with 0.625 mL of 50% v/v propan-1-ol containing 1% w/v dithiothreitol (10 mg/mL) at 60°C for 30 minutes with vortex agitation every 5 minutes. Tubes were centrifuged at 20,000g for 10 minutes and a 0.5 mL aliquot was removed from each tube and transferred to a fresh Eppendorf tube. The high molecular weight glutenins were precipitated in the following way. While gently agitating on a vortex mixer, 0.125 mL of propan-1-ol was added to bring the final propan-1-ol concentration to 60%(v/v). The mixture was capped and vortexed vigorously and then allowed to stand at room temperature (21°C) for 30 minutes. After this time, the precipitate was collected by centrifugation at 3000g for 5 minutes. The tubes were freeze-dried overnight and the resulting protein pooled prior to storage in the freezer at -10°C.

7.10.2 Reaction of the purified high molecular weight glutenin subunit with TGA

The most suitable concentration of the HMW glutenin was 0.33 mg / mL. 30 μ L of this solution was placed into each of several Eppendorf tubes and incubated in a 37°C water bath for 30 minutes. The appropriate enzyme concentration was also incubated alongside in the same water bath to ensure the same incubation conditions. At the end of 30 minutes, one tube was removed and 30 μ L of the reducing agent mercaptoethanol was added to its contents. This was labelled as the control, placed on ice and subsequently transferred to a -20°C freezer. 30 μ L of the prepared enzyme solution was added to each of the remaining tubes and incubation carried out for the required length of time. Thorough vortex mixing of the tubes was carried out at regular intervals. At the end of each incubation period, 30 μ L of mercaptoethanol was added to quench the reaction. The samples were mixed, placed immediately on ice and transferred to -20°C for storage until analysis by SDS-PAGE.

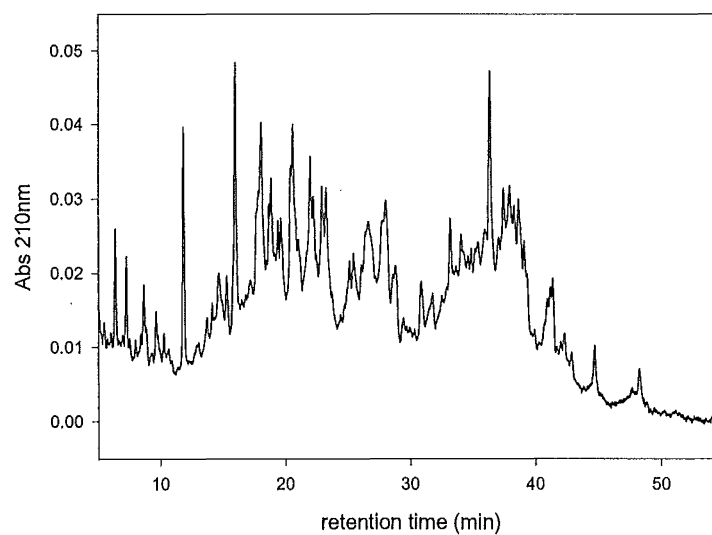
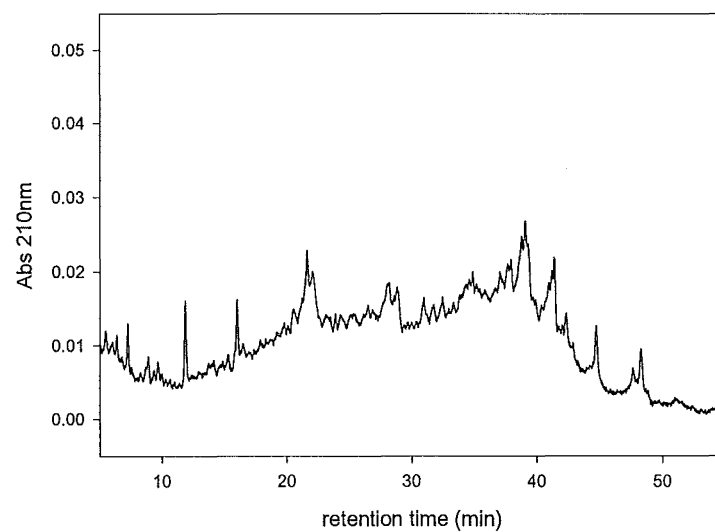
7.11 References

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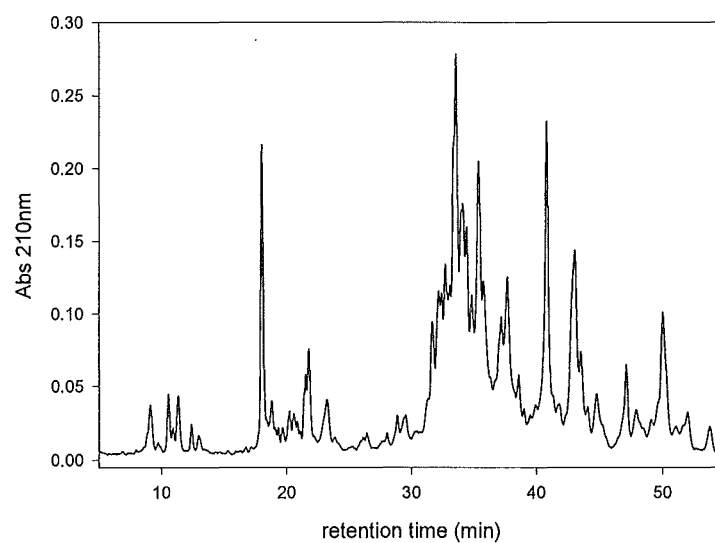
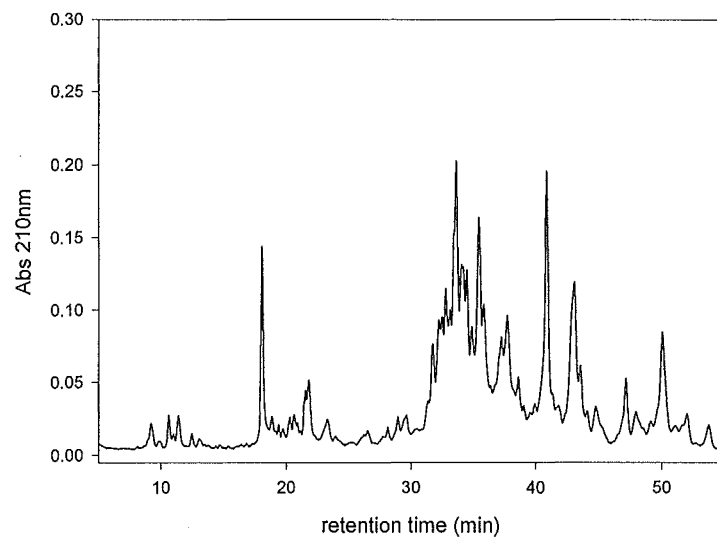
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Appendix

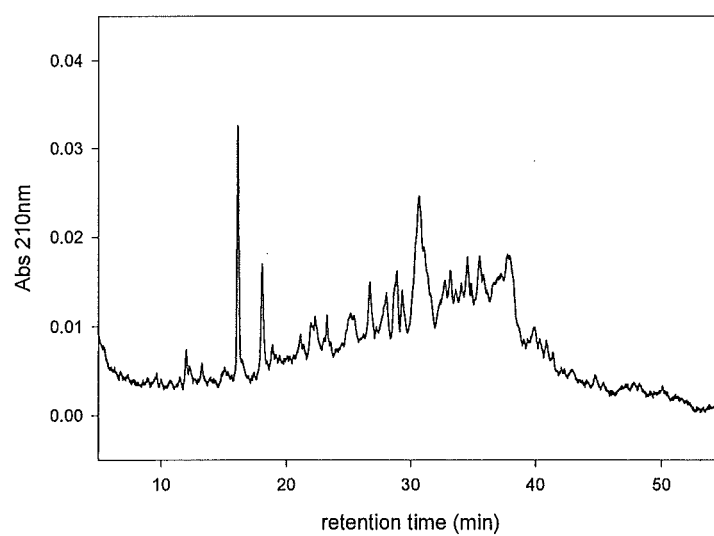
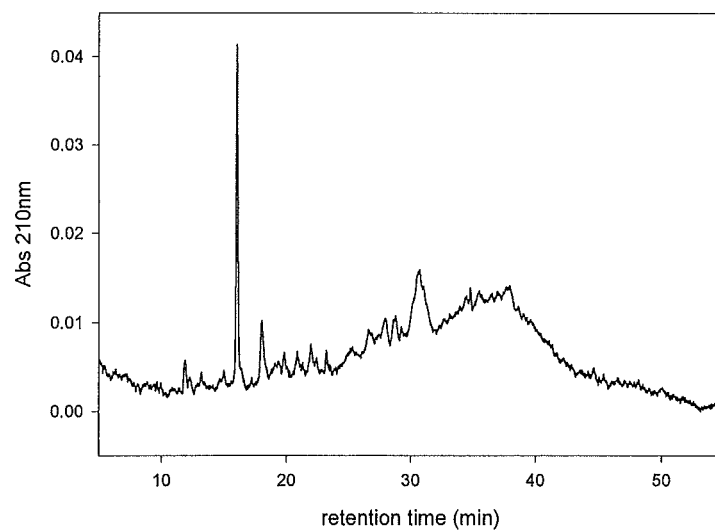
RP-HPLC traces of the albumin / globulin fraction from TGA-treated (upper) and control (lower) croissant doughs, frozen immediately upon preparation.



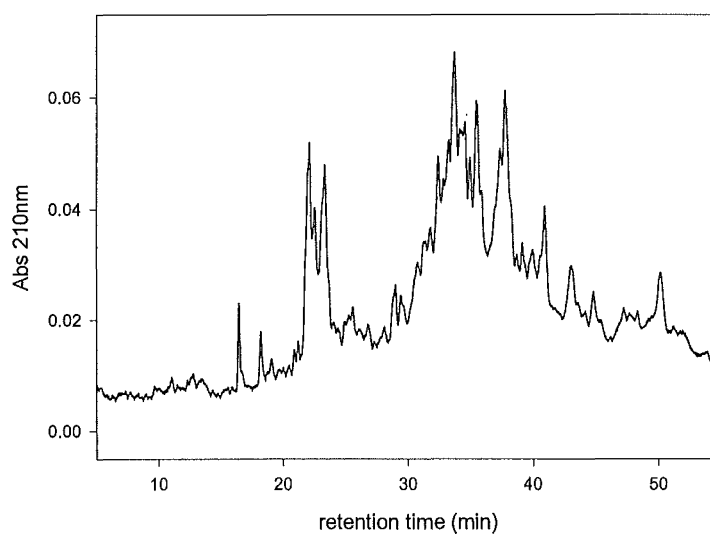
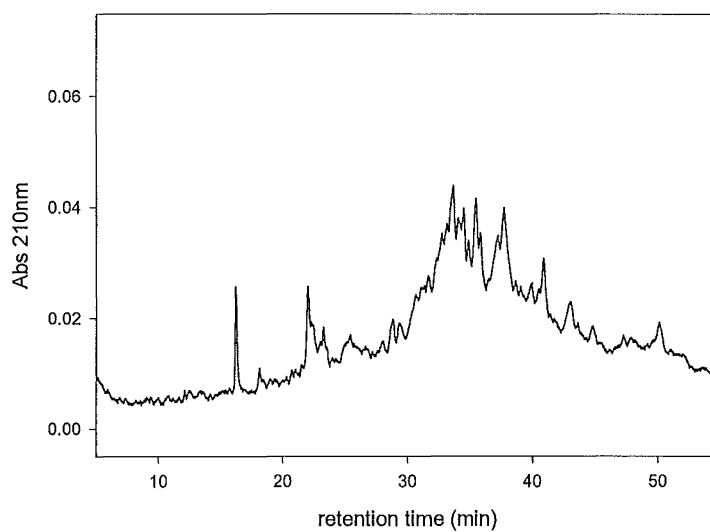
RP-HPLC traces of the gliadin fraction from TGA-treated (upper) and control (lower) croissant doughs, frozen immediately upon preparation.



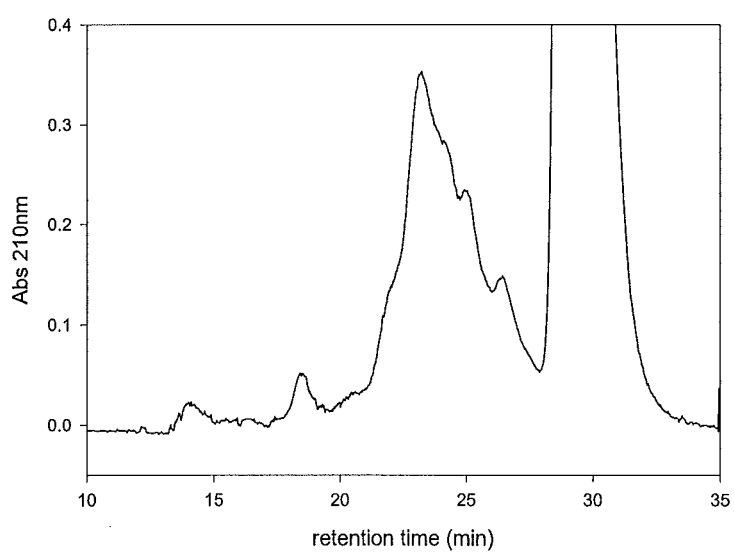
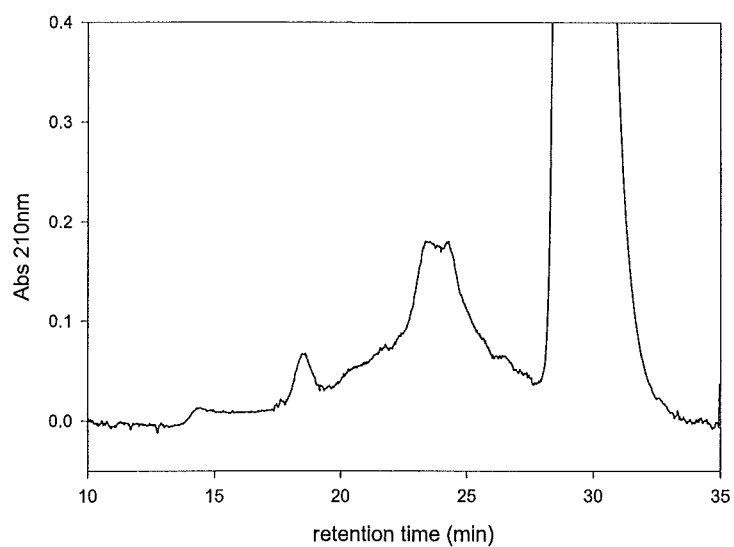
RP-HPLC traces of the SDS-soluble glutenin fraction from TGA-treated (upper) and control (lower) croissant doughs, frozen immediately upon preparation.



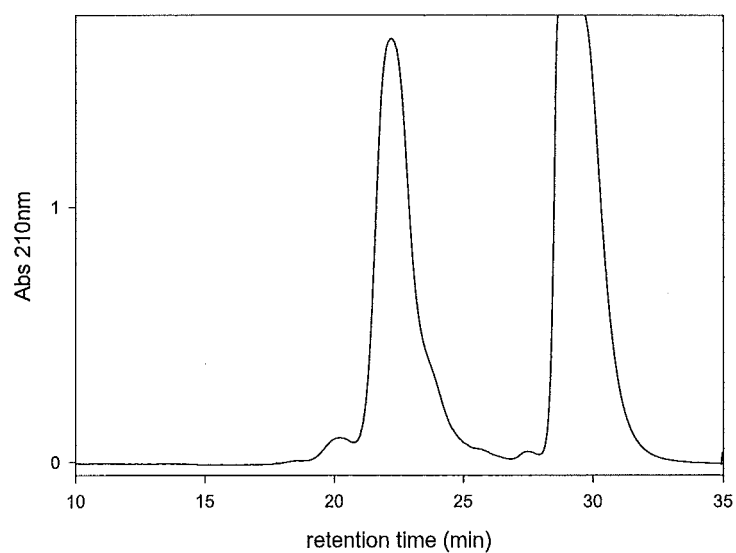
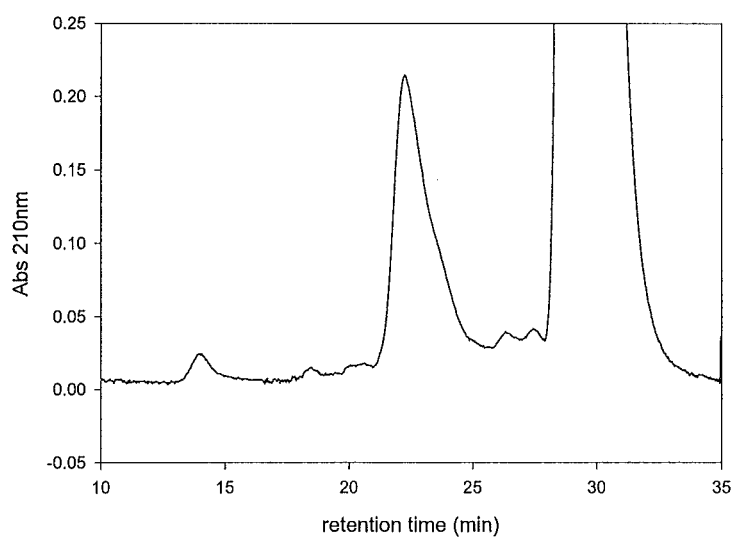
RP-HPLC traces of the SDS-insoluble glutenin fraction from TGA-treated (upper) and control (lower) croissant doughs, frozen immediately upon preparation.



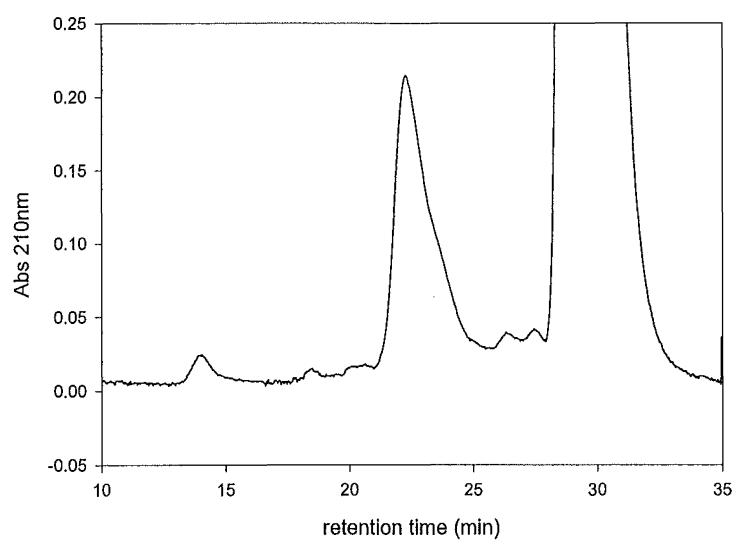
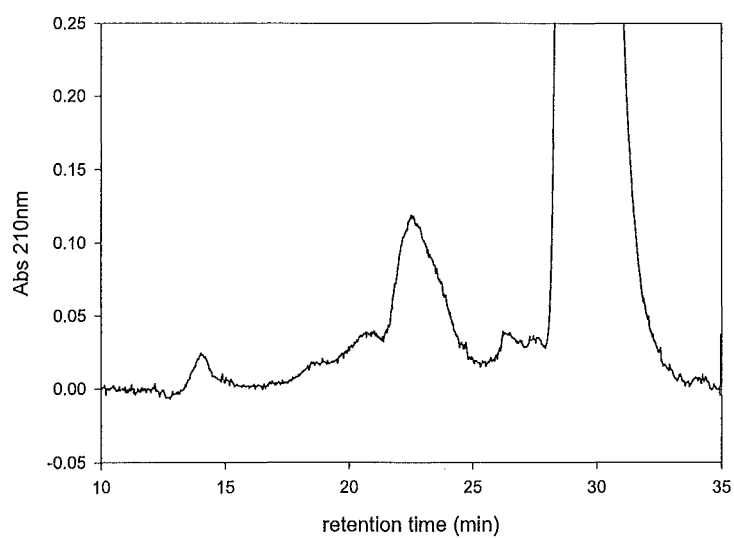
SE-HPLC traces of the albumin / globulin fraction from TGA-treated (upper) and control (lower) croissant doughs, frozen immediately upon preparation.



SE-HPLC traces of the gliadin fraction from TGA-treated (upper) and control (lower) croissant doughs, frozen immediately upon preparation.



SE-HPLC traces of the SDS-soluble glutenin fraction from TGA-treated (upper) and control (lower) croissant doughs, frozen immediately upon preparation.



SE-HPLC traces of the SDS-insoluble glutenin fraction from TGA-treated (upper) and control (lower) croissant doughs, frozen immediately upon preparation.

